Freeform Search

Database: US Pre-Grant Publication Full-Text Database; US Patents Full-Text Database; US OCR Full-Text Database; EPO Abstracts Database; JPO Abstracts Database; Derwent World Patents Index; IBM Technical Disclosure Bulletins

Term: L6 same (11 or 118 or 144 or 161)

Display: 50 **Documents in Display Format:** - **Starting with Number** 1

Generate: ○ Hit List ◉ Hit Count ○ Side by Side ○ Image

Search Clear Interrupt

Search History

DATE: Saturday, February 04, 2006 Printable Copy Create Case

Set Name side by side	Query	Hit Count	Set Name result set
DB=PGPB,USPT,USOC,EPAB,JPAB,DWPI; PLUR=YES; OP=ADJ			
L7	L6 same (11 or 118 or 144 or 161)	38	L7
L6	xylanase same (variant or mutant)	369	L6
L5	L4 and xylanase	7	L5
L4	Sung wing.in.	12	L4
L3	Sung.in.	23264	L3
L2	wing-sung.in.	0	L2
L1	sung-wing.in.	0	L1

END OF SEARCH HISTORY

Hit List

First Hit | Clear | Generate Collection | Print | Fwd Refs | Bkwd Refs | Generate OACS

Search Results - Record(s) 1 through 7 of 7 returned.

Sung et al. applications

☐ 1. Document ID: US 20030166236 A1

L5: Entry 1 of 7 File: PGPB Sep 4, 2003

PGPUB-DOCUMENT-NUMBER: 20030166236
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030166236 A1

TITLE: Modified xylanases exhibiting increased thermophilicity and alkalophilicity

PUBLICATION-DATE: September 4, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Sung, Wing L.	Ontario		CA

US-CL-CURRENT: 435/200

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

☐ 2. Document ID: US 5866408 A

L5: Entry 2 of 7 File: USPT Feb 2, 1999

US-PAT-NO: 5866408
DOCUMENT-IDENTIFIER: US 5866408 A

TITLE: Modification of xylanase to improve thermophilicity, alkophilicity and thermostability

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

☐ 3. Document ID: US 5759840 A

L5: Entry 3 of 7 File: USPT Jun 2, 1998

US-PAT-NO: 5759840
DOCUMENT-IDENTIFIER: US 5759840 A

TITLE: Modification of xylanase to improve thermophilicity, alkalophilicity and thermostability

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

☐ 4. Document ID: US 5405769 A

L5: Entry 4 of 7 File: USPT Apr 11, 1995

US-PAT-NO: 5405769
DOCUMENT-IDENTIFIER: US 5405769 A

TITLE: Construction of thermostable mutants of a low molecular mass xylanase

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 5. Document ID: JP 10179155 A

L5: Entry 5 of 7 File: JPAB Jul 7, 1998

PUB-NO: JP410179155A
DOCUMENT-IDENTIFIER: JP 10179155 A
TITLE: MODIFICATION OF XYLANASE FOR IMPROVING THERMOPHILE, ALKALOPHILICITY AND THERMAL STABILITY

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 6. Document ID: WO 3046169 A2

L5: Entry 6 of 7 File: EPAB Jun 5, 2003

PUB-NO: WO003046169A2
DOCUMENT-IDENTIFIER: WO 3046169 A2
TITLE: XYLANASES WITH ENHANCED THERMOPHILICITY AND ALKALOPHILICITY

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 7. Document ID: EP 828002 A2

L5: Entry 7 of 7 File: EPAB Mar 11, 1998

PUB-NO: EP000828002A2
DOCUMENT-IDENTIFIER: EP 828002 A2
TITLE: Modification of xylanase to improve thermophilicity, alkophilicity and thermostability

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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Clear | Generate Collection | Print | Fwd Refs | Bkwd Refs | Generate OACS

Term	Documents
XYLANASE	2659
XYLANASES	1452
(4 AND XYLANASE).PGPB,USPT,USOC,EPAB,JPAB,DWPI.	7
(L4 AND	

L5: Entry 7 of 7 File: EPAB Mar 11, 1998

PUB-NO: EP000828002A2
DOCUMENT-IDENTIFIER: EP 828002 A2
TITLE: Modification of xylanase to improve thermophilicity, alkophilicity and thermostability

PUBN-DATE: March 11, 1998

INVENTOR-INFORMATION:

NAME	COUNTRY
SUNG, WING L	CA
YAGUCHI, MAKOTO	CA
ISHIKAWA, KAZUHIKO	JP

INT-CL (IPC): C12 N 15/56; C12 N 9/24; D21 C 9/10; C12 N 15/62
EUR-CL (EPC): C12N009/24; D21C005/00

ABSTRACT:

CHG DATE=19990617 STATUS=O> Producing a xylanase enzyme of superior performance in the bleaching of pulp. More specifically, a modified xylanase of Family 11 that shows improved thermophilicity, alkalophilicity, and thermostability as compared to the natural xylanase. The modified xylanases contain any of three types of modifications: (1) changing amino acids 10, 27, and 29 of Trichoderma reesei xylanase II or the corresponding amino acids of another Family 11 xylanase, where these amino acids are changed to histidine, methionine, and leucine, respectively; (2) substitution of amino acids in the N-terminal region with amino acids from another xylanase enzyme. In a preferred embodiment, substitution of the natural Bacillus circulans or Trichoderma reesei xylanase with a short sequence of amino acids from Thermomonospora fusca xylanase yielded chimeric xylanases with higher thermophilicity and alkalophilicity; (3) an extension upstream of the N-terminus of up to 10 amino acids. In a preferred embodiment, extension of the N-terminus of the xylanase with the tripeptide glycine-arginine-arginine improved its performance.

Hit List

First Hit | Clear | Generate Collection | Print | Fwd Refs | Bkwd Refs | Generate OACS

Search Results - Record(s) 1 through 38 of 38 returned.

☐ 1. Document ID: US 20060003433 A1

L7: Entry 1 of 38 File: PGPB Jan 5, 2006

PGPUB-DOCUMENT-NUMBER: 20060003433
PGPUB-FILING-TYPE:
DOCUMENT-IDENTIFIER: US 20060003433 A1

TITLE: Xylanases, nucleic acids encoding them and methods for making and using them

PUBLICATION-DATE: January 5, 2006

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Steer; Brian	San Diego	CA	US
Callen; Walter	San Diego	CA	US
Healey; Shaun	Carlsbad	CA	US
Hazlewood; Geoff	San Diego	CA	US
Wu; Di	San Diego	CA	US
Blum; David	San Diego	CA	US
Esteghlalian; Alireza	La Jolla	CA	US

US-CL-CURRENT: 435/209; 435/320.1, 435/325, 435/69.1, 536/23.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

☐ 2. Document ID: US 20050271769 A1

L7: Entry 2 of 38 File: PGPB Dec 8, 2005

PGPUB-DOCUMENT-NUMBER: 20050271769
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20050271769 A1

TITLE: Enzyme

PUBLICATION-DATE: December 8, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Sibbesen, Ole	Bagsvaerd		DK
Sorensen, Jens Frisbaek	Aarbus		DK

US-CL-CURRENT: 426/20; 435/101, 435/200, 435/252.31, 435/320.1, 435/69.1, 536/23.2

☐ 3. Document ID: US 20050257905 A1

L7: Entry 3 of 38 File: PGPB Nov 24, 2005

PGPUB-DOCUMENT-NUMBER: 20050257905
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20050257905 A1

TITLE: Process and composition for preparing a lignocellulose-based product, and the product obtained by the process

PUBLICATION-DATE: November 24, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Shoseyov, Oded	Karme Yossef		IL

US-CL-CURRENT: 162/72; 162/158, 162/164.1, 435/101

☐ 4. Document ID: US 20050210548 A1

L7: Entry 4 of 38 File: PGPB Sep 22, 2005

PGPUB-DOCUMENT-NUMBER: 20050210548
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20050210548 A1

TITLE: Polypeptides having xylanase activity and polynucleotides encoding same

PUBLICATION-DATE: September 22, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Yaver, Debbie	Davis	CA	US
Harris, Paul	Carnation	WA	US
Otani, Suzanne	Elk Grove	CA	US
Lin, Janine	Davis	CA	US
Ge, Haiyan	Davis	CA	US

US-CL-CURRENT: 800/284; 435/200, 435/419, 435/468, 435/6, 435/69.1, 536/23.2

☐ 5. Document ID: US 20050150619 A1

L7: Entry 5 of 38 File: PGPB Jul 14, 2005

PGPUB-DOCUMENT-NUMBER: 20050150619
PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20050150619 A1

TITLE: Xylanase treatment of chemical pulp

PUBLICATION-DATE: July 14, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Tolan, Jeffrey S.	Ontario		CA
Popovici, Corina	Ontario		CA

US-CL-CURRENT: 162/72; 162/74, 162/78, 435/277, 435/278

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 6. Document ID: US 20050079573 A1

L7: Entry 6 of 38 File: PGPB Apr 14, 2005

PGPUB-DOCUMENT-NUMBER: 20050079573
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20050079573 A1

TITLE: Proteins

PUBLICATION-DATE: April 14, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Sibbesen, Ole	Bagsvaerd		DK

US-CL-CURRENT: 435/69.1; 435/200, 435/254.21, 435/320.1, 536/23.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 7. Document ID: US 20050014233 A1

L7: Entry 7 of 38 File: PGPB Jan 20, 2005

PGPUB-DOCUMENT-NUMBER: 20050014233
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20050014233 A1

TITLE: Genes coding carbon metabolism and energy-producing proteins

PUBLICATION-DATE: January 20, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Zelder, Oskar	Speyer		DE
Pompejus, Markus	Freinsheim		DE
Schroder, Hartwig	Nussloch		DE
Kroger, Burkhard	Limburgerhof		DE

Klopprogge, Corinna	Ludwigshafen	DE
Haberhauer, Gregor	Limburgerhof	DE

US-CL-CURRENT: 435/115; 435/193, 435/320.1, 435/325, 435/69.1, 536/23.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 8. Document ID: US 20040234998 A1

L7: Entry 8 of 38 File: PGPB Nov 25, 2004

PGPUB-DOCUMENT-NUMBER: 20040234998
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20040234998 A1

TITLE: Proteins

PUBLICATION-DATE: November 25, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Sibbesen, Ole	Bagsvaerd		DK
Sorensen, Jens Frisbaek	Aarhus		DK

US-CL-CURRENT: 435/6; 435/320.1, 435/325, 435/69.1, 530/350, 536/23.5

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 9. Document ID: US 20040112555 A1

L7: Entry 9 of 38 File: PGPB Jun 17, 2004

PGPUB-DOCUMENT-NUMBER: 20040112555
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20040112555 A1

TITLE: Bleaching stage using xylanase with hydrogen peroxide, peracids, or a combination thereof

PUBLICATION-DATE: June 17, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Tolan, Jeffrey	Ontario		CA
Suchy, Miro	Ontario		CA

US-CL-CURRENT: 162/72; 162/65, 162/76, 162/78, 162/88, 162/89

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 10. Document ID: US 20040077071 A1

L7: Entry 10 of 38 File: PGPB Apr 22, 2004

PGPUB-DOCUMENT-NUMBER: 20040077071
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20040077071 A1

TITLE: Methods of xylanase treatment in bleaching

PUBLICATION-DATE: April 22, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Tolan, Jeffrey S.	Ontario		CA
Popovici, Corina	Ottawa Ontario		CA
Thibault, Luc	Ontario		CA

US-CL-CURRENT: 435/278; 162/73, 162/78

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 11. Document ID: US 20040014242 A1

L7: Entry 11 of 38 File: PGPB Jan 22, 2004

PGPUB-DOCUMENT-NUMBER: 20040014242
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20040014242 A1

TITLE: Process for immobilizing orientation-controlled protein and process for arraying and immobilizing protein using the same

PUBLICATION-DATE: January 22, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Iwakura, Masahiro	Ibaraki		JP
Hirota, Kiyonori	Ibaraki		JP

US-CL-CURRENT: 436/528

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 12. Document ID: US 20040002136 A1

L7: Entry 12 of 38 File: PGPB Jan 1, 2004

PGPUB-DOCUMENT-NUMBER: 20040002136
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20040002136 A1

TITLE: Transformation system in the field of filamentous fungal hosts

PUBLICATION-DATE: January 1, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Emalfarb, Mark Aaron	Jupiter	FL	US
Burlingame, Richard Paul	Manitowoc	WI	US
Olson, Philip Terry	Manitowoc	WI	US
Sinitsyn, Arkady Panteleimonovich	Moscow	WI	RU
Parriche, Martine	Toulouse		FR
Bousson, Jean Christophe	Quint-Fonsegrives		FR
Pynnonen, Christine Marie	Appleton		US
Punt, Peter Jan	Houten		NL
Van Zeijl, Cornelia Maria Johanna	Vleuten-De Meern		NL

US-CL-CURRENT: 435/69.1; 435/189, 435/193, 435/196, 435/200, 435/219, 435/254.2, 435/320.1, 536/23.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 13. Document ID: US 20030224379 A1

L7: Entry 13 of 38 File: PGPB Dec 4, 2003

PGPUB-DOCUMENT-NUMBER: 20030224379
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030224379 A1

TITLE: Novel nucleic acids and polypeptides

PUBLICATION-DATE: December 4, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Tang, Y. Tom	San Jose	CA	US
Yang, Yonghong	San Jose	CA	US
Wang, Zhiwei	Sunnyvale	CA	US
Weng, Gezhi	Piedmont	CA	US
Ma, Yunqing	Santa Clara	CA	US

US-CL-CURRENT: 435/6; 435/183, 435/320.1, 435/325, 435/69.1, 530/350, 536/23.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☑ 14. Document ID: US 20030180895 A1

L7: Entry 14 of 38 File: PGPB Sep 25, 2003

PGPUB-DOCUMENT-NUMBER: 20030180895
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030180895 A1

TITLE: Xylanse variants having altered sensitivity to xylanse inhibitors

PUBLICATION-DATE: September 25, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Sibbesen, Ole	Bagsvaerd		DK
Sorensen, Jens Frisbaek	Aarbus		DK

US-CL-CURRENT: 435/101; 435/200, 435/320.1, 435/419, 435/69.1, 536/23.2, 800/284

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 15. Document ID: US 20030166236 A1

L7: Entry 15 of 38 File: PGPB Sep 4, 2003

PGPUB-DOCUMENT-NUMBER: 20030166236
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030166236 A1

TITLE: Modified xylanases exhibiting increased thermophilicity and alkalophilicity

PUBLICATION-DATE: September 4, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Sung, Wing L.	Ontario		CA

US-CL-CURRENT: 435/200

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 16. Document ID: US 6936703 B2

L7: Entry 16 of 38 File: USPT Aug 30, 2005

US-PAT-NO: 6936703
DOCUMENT-IDENTIFIER: US 6936703 B2

TITLE: Biocatalyst inhibitors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw De

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☐ 17. Document ID: US 6914043 B1

L7: Entry 17 of 38 File: USPT Jul 5, 2005

US-PAT-NO: 6914043
DOCUMENT-IDENTIFIER: US 6914043 B1

TITLE: Frozen food products comprising anti-freeze protein (AFP) type III HPLC 12

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw De

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☐ 18. Document ID: US 6855365 B2

L7: Entry 18 of 38 File: USPT Feb 15, 2005

US-PAT-NO: 6855365
DOCUMENT-IDENTIFIER: US 6855365 B2

TITLE: Recombinant bacterial phytases and uses thereof

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw De

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☐ 19. Document ID: US 6734344 B2

L7: Entry 19 of 38 File: USPT May 11, 2004

US-PAT-NO: 6734344
DOCUMENT-IDENTIFIER: US 6734344 B2

TITLE: Coniothyrium minitans .beta.-(1,3) exoglucanase gene cbeg 1

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw De

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☐ 20. Document ID: US 6682923 B1

L7: Entry 20 of 38 File: USPT Jan 27, 2004

US-PAT-NO: 6682923
DOCUMENT-IDENTIFIER: US 6682923 B1

TITLE: Thermostable alkaliphilic xylanase

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw De

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☐ 21. Document ID: US 6573086 B1

L7: Entry 21 of 38 File: USPT Jun 3, 2003

US-PAT-NO: 6573086
DOCUMENT-IDENTIFIER: US 6573086 B1
**** See image for Certificate of Correction ****

TITLE: Transformation system in the field of filamentous fungal hosts

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw De

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☐ 22. Document ID: US 6387666 B1

L7: Entry 22 of 38 File: USPT May 14, 2002

US-PAT-NO: 6387666
DOCUMENT-IDENTIFIER: US 6387666 B1

TITLE: High molecular weight pullulan and method for its production

☐ 23. Document ID: US 6331416 B1

L7: Entry 23 of 38 File: USPT Dec 18, 2001

US-PAT-NO: 6331416
DOCUMENT-IDENTIFIER: US 6331416 B1

TITLE: Process of expressing and isolating recombinant proteins and recombinant protein products from plants, plant derived tissues or cultured plant cells

☐ 24. Document ID: US 6228983 B1

L7: Entry 24 of 38 File: USPT May 8, 2001

US-PAT-NO: 6228983
DOCUMENT-IDENTIFIER: US 6228983 B1
** See image for Certificate of Correction **

TITLE: Human respiratory syncytial virus peptides with antifusogenic and antiviral activities

☐ 25. Document ID: US 6093794 A

L7: Entry 25 of 38 File: USPT Jul 25, 2000

US-PAT-NO: 6093794
DOCUMENT-IDENTIFIER: US 6093794 A

TITLE: Isolated peptides derived from the Epstein-Barr virus containing fusion inhibitory domains

☐ 26. Document ID: US 6060065 A

L7: Entry 26 of 38 File: USPT May 9, 2000

US-PAT-NO: 6060065
DOCUMENT-IDENTIFIER: US 6060065 A

TITLE: Compositions for inhibition of membrane fusion-associated events, including influenza virus transmission

☐ 27. Document ID: US 6010899 A

L7: Entry 27 of 38 File: USPT Jan 4, 2000

US-PAT-NO: 6010899
DOCUMENT-IDENTIFIER: US 6010899 A

TITLE: High molecular weight pullulan and method for its production

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 28. Document ID: US 5871730 A

L7: Entry 28 of 38 File: USPT Feb 16, 1999

US-PAT-NO: 5871730
DOCUMENT-IDENTIFIER: US 5871730 A
** See image for Certificate of Correction **

TITLE: Thermostable xylanase DNA, protein and methods of use

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 29. Document ID: US 5866408 A

L7: Entry 29 of 38 File: USPT Feb 2, 1999

US-PAT-NO: 5866408
DOCUMENT-IDENTIFIER: US 5866408 A

TITLE: Modification of xylanase to improve thermophilicity, alkophilicity and thermostability

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 30. Document ID: US 5759840 A

L7: Entry 30 of 38 File: USPT Jun 2, 1998

US-PAT-NO: 5759840
DOCUMENT-IDENTIFIER: US 5759840 A

TITLE: Modification of xylanase to improve thermophilicity, alkalophilicity and thermostability

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

---

☐ 31. Document ID: US 5705379 A

L7: Entry 31 of 38 File: USPT Jan 6, 1998

US-PAT-NO: 5705379
DOCUMENT-IDENTIFIER: US 5705379 A

TITLE: Nucleotide sequences encoding a thermostable alkaline protease

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 32. Document ID: US 5677161 A

L7: Entry 32 of 38 File: USPT Oct 14, 1997

US-PAT-NO: 5677161
DOCUMENT-IDENTIFIER: US 5677161 A

TITLE: Preparation exhibiting enzymatic activity, a method of producing the same, and applications thereof

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 33. Document ID: US 5434071 A

L7: Entry 33 of 38 File: USPT Jul 18, 1995

US-PAT-NO: 5434071
DOCUMENT-IDENTIFIER: US 5434071 A

TITLE: .alpha.-L-arabinofuranosidase and xylanase from Bacillus stearothermophilus NCIMB 40221, NCIMB 40222 or mutant thereof for delignification

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 34. Document ID: US 5405769 A

L7: Entry 34 of 38 File: USPT Apr 11, 1995

US-PAT-NO: 5405769
DOCUMENT-IDENTIFIER: US 5405769 A

TITLE: Construction of thermostable mutants of a low molecular mass xylanase

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 35. Document ID: US 5268460 A

L7: Entry 35 of 38 File: USPT Dec 7, 1993

US-PAT-NO: 5268460
DOCUMENT-IDENTIFIER: US 5268460 A

TITLE: High molecular weight pullulan

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 36. Document ID: US 5116746 A

L7: Entry 36 of 38 File: USPT May 26, 1992

US-PAT-NO: 5116746
DOCUMENT-IDENTIFIER: US 5116746 A

TITLE: Cellulase-free endo-xylanase enzyme of use in pulp delignification

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 37. Document ID: CN 1132929 C, CN 1333371 A

L7: Entry 37 of 38 File: DWPI Dec 31, 2003

DERWENT-ACC-NO: 2002-305620
DERWENT-WEEK: 200564

TITLE: Enzyme method preparation for high-purity oligoxylose

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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☐ 38. Document ID: US 3813318 A

L7: Entry 38 of 38 File: USOC May 28, 1974

US-PAT-NO: 3813318
DOCUMENT-IDENTIFIER: US 3813318 A

TITLE: PRODUCTION OF XYLOSE (DEXTROSE) ISOMERASE ENZYME PREPARATIONS

DATE-ISSUED: May 28, 1974

INVENTOR-NAME: HEADY R; ARMBRUSTER F ; CORY R

US-CL-CURRENT: 435/94, 435/174, 435/233, 435/448, 435/886

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

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Clear | Generate Collection | Print | Fwd Refs | Bkwd Refs | Generate OACS

Term	Documents
"11"	8860443
11S	4507
"118"	867835
118S	236
"144"	718155
144S	160
"161"	375509
161S	162

(6 SAME ("161" OR "144" OR "118" OR "11")).PGPB,USPT,USOC,EPAB,JPAB,DWPI.	38
(L6 SAME (11 OR 118 OR 144 OR 161)).PGPB,USPT,USOC,EPAB,JPAB,DWPI.	38

Display Format: - Change Format

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US005866408A

United States Patent [19]

Sung et al.

[11] Patent Number: 5,866,408

[45] Date of Patent: Feb. 2, 1999

[54] **MODIFICATION OF XYLANASE TO IMPROVE THERMOPHILICITY, ALKOPHILICITY AND THERMOSTABILITY**

[75] Inventors: **Wing L. Sung**, Gloucester; **Makoto Yaguchi**, Ottawa, both of Canada; **Kazuhiko Ishikawa**, Tsukuba, Japan

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Related U.S. Application Data

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[51] **Int. Cl.**[6] **C12N 9/24**; D21C 3/00

[52] **U.S. Cl.** **435/278**; 435/200

[58] **Field of Search** 435/200, 278

[56] **References Cited**

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[57] **ABSTRACT**

Producing a xylanase enzyme of superior performance in the bleaching of pulp. More specifically, a modified xylanase of Family 11 that shows improved thermophilicity, alkalophilicity, and thermostability as compared to the natural xylanase. The modified xylanases contain any of three types of modifications: (1) changing amino acids 10, 27, and 29 of *Trichoderma reesei* xylanase II or the corresponding amino acids of another Family 11 xylanase, where these amino acids are changed to histidine, methionine, and leucine, respectively; (2) substitution of amino acids in the N-terminal region with amino acids from another xylanase enzyme. In a preferred embodiment, substitution of the natural *Bacillus circulans* or *Trichoderma reesei* xylanase with a short sequence of amino acids from *Thermomonospora fusca* xylanase yielded chimeric xylanases with higher thermophilicity and alkalophilicity; (3) an extension upstream of the N-terminus of up to 10 amino acids. In a preferred embodiment, extension of the N-terminus of the xylanase with the tripeptide glycine-arginine-arginine improved its performance.

8 Claims, 26 Drawing Sheets

Claims are drawn to method of improving bleachability using a mutant

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L5: Entry 6 of 7 File: EPAB Jun 5, 2003

PUB-NO: WO003046169A2
DOCUMENT-IDENTIFIER: WO 3046169 A2
TITLE: XYLANASES WITH ENHANCED THERMOPHILICITY AND ALKALOPHILICITY

PUBN-DATE: June 5, 2003 , filed 11/20/02 - priority to US 09/990874

INVENTOR-INFORMATION:

NAME	COUNTRY
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INT-CL (IPC): C12 N 9/24; D21 H 17/00
EUR-CL (EPC): C12N009/24; D21C009/10

ABSTRACT:

CHG DATE=20040424 STATUS=O>The present invention provides a xylanase, or a modified xylanase enzyme comprising at least one substituted amino acid residue at a position selected from the group consisting of amino acid 11, 116, 118, 144 and 161, the position determined from sequence alignment of the modified xylanase with

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L7: Entry 2 of 38 File: PGPB Dec 8, 2005

DOCUMENT-IDENTIFIER: US 20050271769 A1
TITLE: Enzyme

Appl no: 11/170653, Sibbesen et al. — priority to 10/237,386, 9/9/02 which is C-I-P of PCT/IB01/00426 3/8/01.

Summary of Invention Paragraph:
[0016] For some embodiments, preferably the variant polypeptide is derived from a family 11 xylanase.

Summary of Invention Paragraph:
[0034] In one embodiment, the present invention provides a variant xylanase polypeptide or fragment thereof having xylanase activity, comprising one or more amino acid modifications at any one of amino acid residues numbers 11, 12 and 13 of the B. subtilis amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

Summary of Invention Paragraph:
[0036] For some embodiments, preferably the variant xylanase polypeptide, or fragment thereof having xylanase activity, comprises one or more amino acid modifications at any one of amino acid residues numbers: 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 29, 30, 31, 32, 33, 34, 35, 36, 37, 61, 62, 63, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 173, 174, 175, 176, 177, 178 of the B. subtilis amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

Summary of Invention Paragraph:
[0044] The variant xylanase polypeptide may comprise other modifications in other amino acid residues, such as modification at any one of amino acid residues: 1, 2, 46, 47, 48, 49, 50, 51, 52, 53, 54, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 184, 185 of the B. subtilis amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

Detail Description Table CWU:
15TABLE 11 Relative viscosity of flour slurry as a function of xylanase mutant and parent xylanase (here wild type xylanase) Incubation time, minutes Mutant ID 0 2 5 10 20 Relative viscosity change, % Wildtype 100 112 120 131 141 D11Y 100 97 93 83 75 D11N 100 112 125 130 136 D11F 100 93 87 78 69 D11K 100 105 95 88 78 D11S 100 102 110 113 117 D11W 100 106 115 121 122 G34D 100 110 120 128 124 G34F 100 111 126 128 146 G34T 100 100 108 111 106 Y113A 100 118 129 130 124 Y113D 100 116 127 124 114 Y113K 100 118 123 121 115 N114A 100 117 128 127 131 N114D 100 125 144 162 170 N114F 100 113 119 131 150 N114K 100 119 129 141 147 D121N 100 104 103 106 104 D121K 100 122 132 141 162 D121F 100 107 117 128 147 D121A 100 101 102 103 107 R122D 100 120 119 124 115 R122F 100 127 144 150 160 R122A 100 123 138 144 153 Q175E 100 116 134 142 149 Q175S 100 110 113 121 129 Q175L 100 111 111 119 126 G12F 100 127 132 122 101 G13F 100 106 119 124 113 I15K 100 109 108 113 118 N32K 100 97 98 101 101 G120K 100 103 111 115 121 G120D 100 112 122 120 126 G120F 100 103 111 117 130 G120Y 100 106 106 108 126 G120N 100 119 123 130 141 D119K 100 118 119 127 125 D119Y 100 102 102 111 110 D119N 100 126 137 145 146 T123K 100 106 109 121 120 T123Y 100 101 106 108 116 T123D 100 113 123 125 126 T124K 100 117 131 128 127 T124Y 100 112 123 132 135 T124D 100 103 110 111 118 N17K 100 114 119 119 132 N17Y 100 102 102 108 108 N17D 100 120 131 135 143 N29K 100 98 100 100 104 N29Y 100 115 117 132 143 N29D 100 104 104 113 111 S31K 100 119 115 124 134 S31Y 100 110 118 122 137 S31D 100 99 103 109 110 D11F/R122D 100 91 89 82 77 D11F/G34D 100 96 93 84 80

L7: Entry 14 of 38 File: PGPB Sep 25, 2003

DOCUMENT-IDENTIFIER: US 20030180895 A1
TITLE: Xylanse variants having altered sensitivity to xylanse inhibitors

US: 10/237386 9/9/02, Sibbesen etal. priority to PCT/IB01/00428 3/8/01.

Summary of Invention Paragraph:
[0015] For some embodiments, preferably the variant polypeptide is derived from a family 11 xylanase.

Summary of Invention Paragraph:
[0033] In one embodiment, the present invention provides a variant xylanase polypeptide or fragment thereof having xylanase activity, comprising one or more amino acid modifications at any one of amino acid residues numbers 11, 12 and 13 of the B. subtilis amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

Summary of Invention Paragraph:
[0035] For some embodiments, preferably the variant xylanase polypeptide, or fragment thereof having xylanase activity, comprises one or more amino acid modifications at any one of amino acid residues numbers: 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 29, 30, 31, 32, 33, 34, 35, 36, 37, 61, 62, 63, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 173, 174, 175, 176, 177, 178 of the B. subtilis amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

Summary of Invention Paragraph:
[0043] The variant xylanase polypeptide may comprise other modifications in other amino acid residues, such as modification at any one of amino acid residues: 1, 2, 46, 47, 48, 49, 50, 51, 52, 53, 54, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 184, 185 of the B. subtilis amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

Detail Description Table CWU:
14TABLE 11 Relative viscosity of flour slurry as a function of xylanase mutant and parent xylanase (here wild type xylanase) Incubation time, minutes Mutant ID 0 2 5 10 20 Relative viscosity change, % Wildtype 100 112 120 131 141 D11Y 100 97 93 83 75 D11N 100 112 125 130 136 D11F 100 93 87 78 69 D11K 100 105 95 88 78 D11S 100 102 110 113 117 D11W 100 106 115 121 122 G34D 100 110 120 128 124 G34F 100 111 126 128 146 G34T 100 100 108 111 106 Y113A 100 118 129 130 124 Y113D 100 116 127 124 114 Y113K 100 118 123 121 115 N114A 100 117 128 127 131 N114D 100 125 144 162 170 N114F 100 113 119 131 150 N114K 100 119 129 141 147 D121N 100 104 103 106 104 D121K 100 122 132 141 162 D121F 100 107 117 128 147 D121A 100 101 102 103 107 R122D 100 120 119 124 115 R122F 100 127 144 150 160 R122A 100 123 138 144 153 Q175E 100 116 134 142 149 Q175S 100 110 113 121 129 Q175L 100 111 111 119 126 G12F 100 127 132 122 101 G13F 100 106 119 124 113 I15K 100 109 108 113 118 N32K 100 97 98 101 101 G120K 100 103 111 115 121 G120D 100 112 122 120 126 G120F 100 103 111 117 130 G120Y 100 106 106 108 126 G120N 100 119 123 130 141 D119K 100 118 119 127 125 D119Y 100 102 102 111 110 D119N 100 126 137 145 146 T123K 100 106 109 121 120 T123Y 100 101 106 108 116 T123D 100 113 123 125 126 T124K 100 117 131 128 127 T124Y 100 112 123 132 135 T124D 100 103 110 111 118 N17K 100 114 119 119 132 N17Y 100 102 102 108 108 N17D 100 120 131 135 143 N29K 100 98 100 100 104 N29Y 100 115 117 132 143 N29D 100 104 104 113 111 S31K 100 119 115 124 134 S31Y 100 110 118 122 137 S31D 100 99 103 109 110 D11F/R122D 100 91 89 82 77 D11F/G34D 100 96 93 84 80

CLAIMS:

2. A variant polypeptide according to claim 1 which is derived from a family 11 xylanase.

7. A variant xylanase polypeptide, or fragment thereof having xylanase activity, according to any one of the preceding claims wherein said amino acid modification is at any one or more of amino acid residues numbers: 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 122, 123, 124 and 175 of the B. subtilis amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

9. A variant xylanase polypeptide, or fragment thereof having xylanase activity, according to claim 8 wherein said other amino acid residues are any one or more of amino acid residues numbers: 3, 4, 5, 6, 7, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 38, 39, 40, 41, 42, 43, 44, 45, 55, 56, 57, 58, 59, 60, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 108, 109, 110, 126, 127, 128, 129, 130, 131, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 179, 180, 181, 182, 183 of the B. subtilis amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

10. A variant xylanase polypeptide, or fragment thereof having xylanase activity, according to claim 8 wherein said other surface amino acid residues are any one or more of amino acid residues numbers: 1, 2, 46, 47, 48, 49, 50, 51, 52, 53, 54, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 184, 185 of the B. subtilis amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

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Stabilization of xylanase by random mutagenesis

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Received 2 October 1992; revised version received 2 December 1992

Four heat-resistant mutants of xylanase (N56, N102, N104 and F1) were obtained by random mutagenesis. The mutant genes had the following amino acid changes: N56, Ser-26 to Trp, Gly-38 to Asp and Thr-126 to Ser; N102, Gly-38 to Asp; N104, Gly-38 to Ser and Arg-48 to Lys; F1, Ser-12 to Cys. Kinetic studies showed that N104 is stabilized by an increase in the activation enthalpy, while the other mutants are stabilized by a decrease in the activation entropy.

Xylanase; *Bacillus pumilus*; Random mutagenesis; Enzyme stabilization; Heat-resistant mutant

1. INTRODUCTION

It is generally believed that enzymes have become well-adapted to their physiological environment and are at their optimum state; therefore, most mutations are thought not to improve the properties of enzymes. Accordingly, the improvement of enzymes has been sought by well-designed site-directed mutagenesis rather than by random mutagenesis. With respect to stability, however, enzymes may not be at their optimal state. In fact, we have obtained many stability-increased mutants of glucose dehydrogenase [1,2]. If this is a common feature of enzymes from mesophiles, random mutagenesis should be a strong method for the stabilization of enzymes. In addition, if randomly-occurring stability-increasing mutations are assigned on the three-dimensional structure of an enzyme, they should provide us with valuable information on enzyme stabilization.

Xylanase (EC 3.2.1.8) is a potentially important enzyme for the use of xylan in agricultural wastes. A xylanase gene (*xynA*) was cloned from *Bacillus pumilus* IPO [3], sequenced [4] and the enzyme was crystallized [5], and its structure was determined by X-ray crystallography at 2.2 Å resolution (unpublished results). In this work, the xylanase gene was randomly mutagenized, and four heat-resistant mutants were obtained. The results obtained here clearly show the usefulness of random mutagenesis for the stabilization of enzymes.

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2. EXPERIMENTAL

2.1. *Materials*

Restriction endonucleases and other enzymes for DNA manipulation were purchased from Toyobo Co. Ltd. (Osaka). The following bacterial strains, bacteriophages, and plasmids were used: *Escherichia coli* strains JM103 (*Δ(lac-pro) thi strA supE endA sbcB15 hsdR4* F′ *traD36 proAB lacI*^{q} *lacZ M15*) [6,7] and KP3998 (F$^-$ *hsdS20* (r_B^- m_B^-) *ara-14 proA2 lac I*^{q} *galK2 rpsL20 xyl-5 mlt-1 supE44* λ^-) [8]; M13mp18 and mp19 phages; and plasmid pHIX312 [9] containing *xynA*. *E. coli* KP3998 was a generous gift from Dr. T. Miki (Kyushu University).

2.2. *Random mutagenesis*

pHIX312 [9] was digested with *Hin*cII and *Bam*HI, and the 0.75-kb DNA fragment containing the xylanase gene was inserted into M13mp18 and M13mp19. The single-stranded DNA of the hybrid plasmid was treated at 20°C with hydrazine (for 5–20 min), formic acid (5–20 min), or sodium nitrite (1–3 h) by the method of Myers et al. [10]. The chemically mutagenized single-stranded DNA was annealed with P1 primer and made into a duplex form by reverse transcriptase [10]. The duplex DNA was digested with *Bst*EII and *Bam*HI, the resulting 0.72-kb fragment containing the main part of *xynA* (lacking the 30-bp 5′-terminal sequence of *xynA*) was ligated with pHIX312 that had been digested with *Bst*EII and *Bam*HI to remove its wild-type *xynA* sequence, and *E. coli* KP3998 was transformed with the hybrid plasmid.

2.3. *Purification of xylanases*

E. coli KP3998 cells harboring a plasmid containing the wild-type or each mutant gene of xylanase were grown on LB medium [11]. (1 liter) as described previously [9]. The cells were suspended in 50 mM potassium phosphate buffer (pH 6.5) containing DNase (3 U/ml) and RNase (0.1 U/ml), disrupted with a French pressure cell, and the supernatant was obtained by centrifugation. Each xylanase was purified from the supernatant by stepwise column chromatographies of DEAE-Sepharose CL-6B and CM-Sephadex C-50 as described previously [12]. Xylanase activity was measured at 40°C as described previously [9,12], using oat spelt xylan (Sigma) as a substrate. The wild-type xylanase corresponds to the M-wild xylanase in the previous paper [9]; this enzyme has a methionine residue before the mature sequence, i.e. at the position of −1.

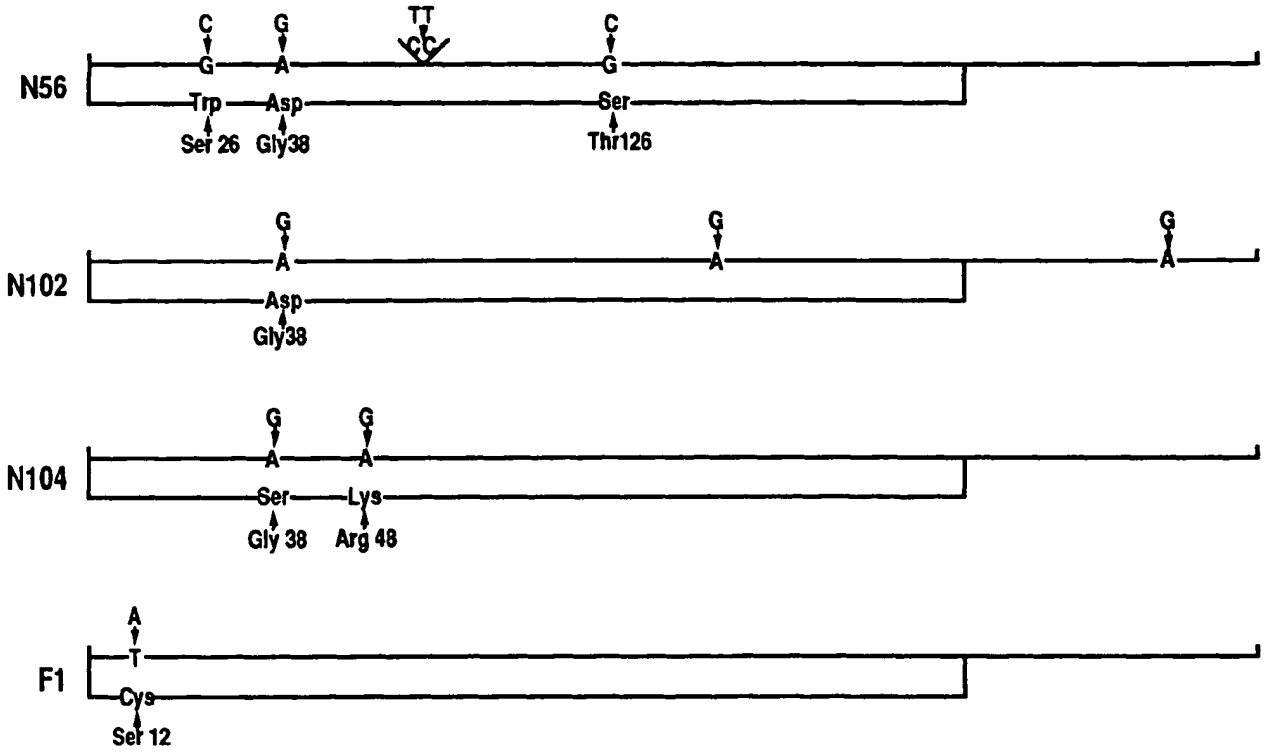

Fig. 1. Base changes and deduced amino acid substitutions in the mutant genes coding for heat-resistant xylanases. The box represents the structural gene of xylanase. We found the following errors in the previous sequence of the wild-type xylanase [4]: adenine at position 336 should be cytosine, adenine at 367 should be guanine, and cytosine at 615 should be thymine; the second error changes the deduced amino acid sequence from Ser-76 to Gly-76.

3. RESULTS

The xylanase gene was chemically mutagenized as described above. The transformants (about 60 000 colonies) harboring the hybrid plasmid containing the mutagenized xylanase gene were analyzed for heat-resistant enzyme activity by heat treatment (60°C for 30 min) followed by Congo red plate assay [13]. The wild-type enzyme is inactivated by the heat treatment and cannot be detected by the plate assay. Among the transformants, we obtained the following four positive clones: N56, N102, N104 and F1. N56, N102, and N104 were obtained by sodium nitrite treatment, and F1 by formic acid treatment.

The nucleotide sequences of the xylanase genes encoding heat-resistant mutant enzymes were identified by a Toyobo Sequence kit (Toyobo Co. Ltd). Fig. 1 shows the base and amino acid substitutions caused by the mutagenesis. The amino acid substitutions cluster in the N-terminal region, although the gene was mutagenized randomly. Especially, the mutation at Gly-38 is observed in all the mutants obtained by the mutagenesis with sodium nitrite. It is also noteworthy that N102 and F1 are stabilized by the following single amino acid substitutions: Gly-38 to Asp and Ser-12 to Cys, respectively. Therefore, Gly-38 and Ser-12 are the main target points for mutations that increase the heat resistance of this enzyme without a large loss of its activity.

The wild type and the four mutants of xylanase (N56, N102, N104, and F1) were purified as described in section 2.3. The homogeneity of the final preparation was checked by SDS-PAGE [14]; the purities of the wild type, N56, N102, N104, and F1 were then quantified with a densitometer to be 100%, 99.9%, 99.9%, 100% and 97.0%, respectively. In addition, these enzymes show a fused single precipitin line with rabbit antiserum against the wild-type enzyme by the double immunodiffusion test [15] (data not shown), thereby confirming the immunological identity of the mutant enzymes.

The effects of the amino acid substitutions in Fig. 1 on the thermostability of xylanase were investigated at various temperatures ranging from 51°C to 61°C, and the results at 57°C are shown in Fig. 2. At the temperatures tested, all the mutant enzymes are more heat-resistant than the wild type, and in the temperature range of less than 59°C, the order is N104 > N56 > N102 > F1. The heat inactivation rate constant (k) was calculated from the results as shown in Fig. 2 assuming first-order kinetics, and the results are shown in Fig. 3. The activation parameters for the heat inactivation were

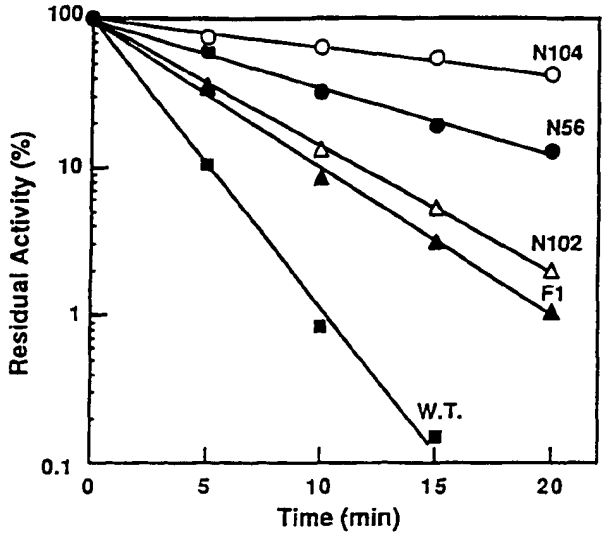

Fig. 2. Time course of heat inactivation of the wild type (W.T.) and mutant xylanases. The enzymes were heated at 57°C in 50 mM potassium phosphate buffer (pH 6.5). The residual activity was measured as described previously [9,12].

estimated from Fig. 3, and the values of the activation enthalpy (ΔH^*) and the activation entropy (ΔS^*) for the mutants are listed in Table I as the difference from those for the wild type. These results show that N104 is stabilized by an increase in ΔH^*, while the other mutants are stabilized by a decrease in ΔS^*. This means that the mechanism of stabilization is different between N104 and the others.

Table I also shows the specific activities of the mutant xylanases relative to that of the wild-type enzyme. The activity of N104 is about 20% of that of the wild type, but those of the other mutants are similar to or even

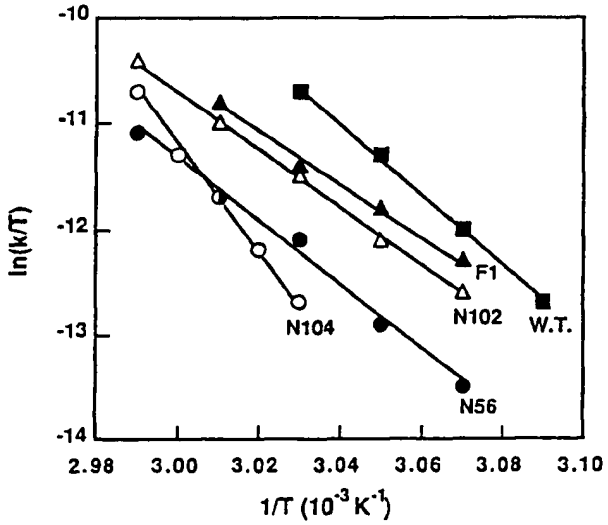

Fig. 3. Arrhenius plot for heat inactivation of the wild-type (W.T.) and mutant xylanases.

higher (N102) than that of the wild-type enzyme. Thus, xylanase was able to be stabilized by random mutagenesis without loosing the catalytic activity.

4. DISCUSSION

Four heat-resistant mutants of xylanase were selected from 60 000 mutant genes prepared by random mutagenesis, and Gly-38 and Ser-12 were assigned as the main target points for stability-increasing mutations. By substituting other amino acid residues at these positions, we will be able to optimize the mutations at these points for the stabilization of this enzyme. It is also important to investigate the additivity of the effects of these mutations including positions other than Gly-38 and Ser-12. In fact, N56 is more heat resistant than N102 owing to the presence of the following two additional mutations: Ser-26 to Trp and Thr-126 to Ser. Thus, random mutagenesis provides us with not only some desired mutants but also many promising strategies for further improvement of the mutants.

Fig. 4 illustrates the positions of the amino acid substitutions found in the heat-resistant mutants on the tertiary structure of xylanase. It is confirmed by computer-graphic simulation that all these substitutions can be accommodated without changing the wild-type conformation. For example, Ser-26 and Gly-38 are on the outer anti-parallel β-sheet, and the substituted residues at these positions protrude from the molecular surface into the solvent without obstructing the motion of the neighboring residues. In addition, it is suggested by the simulation that the carboxyl group of Asp-38 and the hydroxyl group of Ser-189 may form a hydrogen bond in the structures of N56 and N102.

How can we explain the effects of these mutations? For N102, a possible strengthening of the hydrogen bond described above may contribute to the stabilization by increasing ΔH^*. However, the experimental results show that ΔH^* is decreased by the mutation of Gly-38 to Asp, and N102 is stabilized by the decrease

Table I

Specific activity and activation parameters for heat inactivation of mutant xylanases

Enzyme	$\Delta\Delta H^*$ (kJ/mol)	$\Delta\Delta S^*$ (J/mol/K)	Specific activity (%)
N104	+120	+370	19
N56	−30	−100	100
N102	−50	−160	180
F1	−80	−230	100

The activation parameters were calculated from the results shown in Fig. 3, and expressed as the increase (+) or decrease (−) from those of the wild-type enzyme. The values for the wild-type are ΔH^* = 280 kJ/mol and ΔS^* = 560 J/mol/K. The specific activities of the mutants are expressed relative to that of the wild-type enzyme (100% = 241 U/mg).

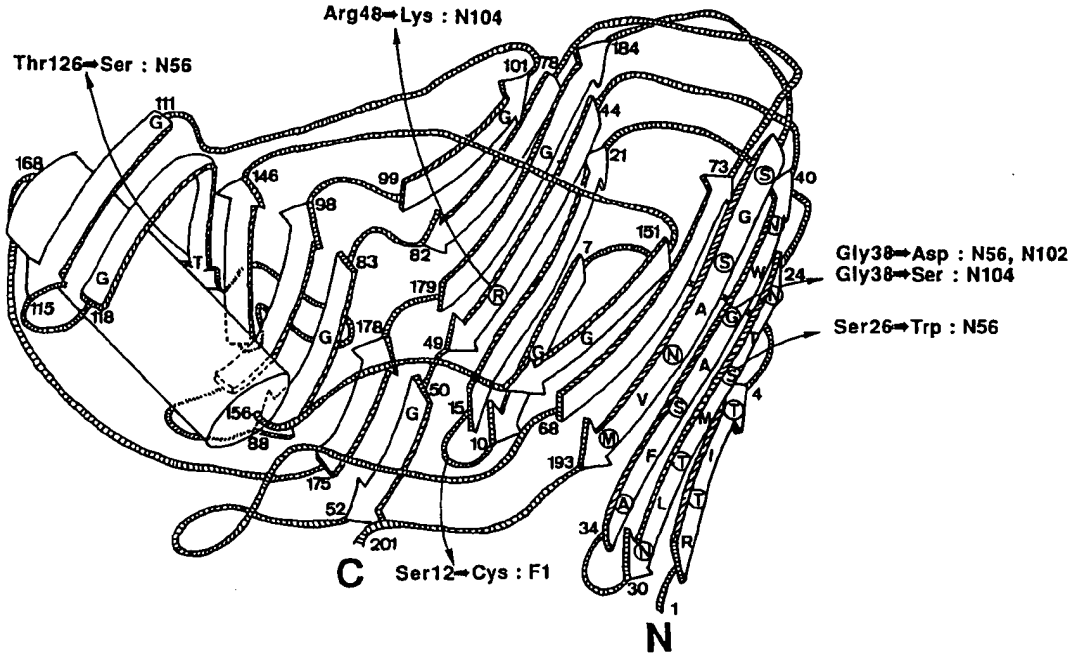

Fig. 4. Schematic illustration of the tertiary structure of xylanase showing the positions of the amino acid substitutions observed in the heat resistant mutants. All the glycine residues on the β-sheets are also shown. The amino acid residues on the β-sheets and protruding from the molecular surface are enclosed with circles.

in ΔS^* (Table I). Therefore, this explanation does not hold true here. On the other hand, Matthews et al. proposed that by replacing glycines at sites that do not interfere with the three-dimensional structure of a protein, it should be possible to decrease the entropy of unfolding of a protein and thereby increase its stability [16,17], and they demonstrated that such substitutions stabilize T4 lysozyme toward reversible and irreversible thermal denaturation [17]. The stabilization of xylanase by the mutation of Gly-38 to Asp (N102) could be explained by this theory, as ΔS^* for N102 is smaller than that for the wild type (Table I). However, the stabilization observed for N104, which contains the mutation of Gly-38 to Ser, is not entropic, and to the contrary, ΔS^* for N104 is larger than that for the wild-type enzyme (Table I). Thus, it is difficult to give a consistent explanation for the stabilizing effects of these mutations, and it is impossible to design these mutations by using the tertiary structure of this enzyme and available theories of stabilization.

The effects of the mutations on the activity of xylanase are also interesting. While the mutation of Ser-12 to Cys (F1) does not affect the activity, that of Gly-38 to Asp (N102) caused an 80% increase. The increased activity is then decreased to the original level by the additional mutations of Ser-26 to Trp and Thr-126 to Ser (N56). As Ser-26 and Gly-38 are neighboring residues on the outer β-sheet, the mutation at Ser-26 may compensate for the effects of the Gly-38 mutation, or as Thr-126 is near the active-site region (including Glu-93 and Glu-182) proposed before [9], the mutation of Thr-126 to Ser may affect the conformation of the region, thereby decreasing the activity. On the other hand, the activity of N104 is only 19% of that of the wild-type enzyme. This decrease in the activity may be mainly due to the mutation of Arg-48 to Lys rather than that of Gly-38 to Ser, because Arg-48 is in the active-site cleft described above.

Although it is difficult to explain the stabilizing effects of these mutations, they may provide us with a general strategy for enzyme stabilization, because there should be some implicit reasons for these four mutants being selected from 60 000 kinds of genes. For example, Gly-38 was selected as a target point out of 24 glycine residues of the xylanase molecule. In addition, this is only one glycine residue that satisfies the following points: it is on a β-sheet, and if it is replaced, the substituted residue protrudes from the molecular surface into the solvent. Therefore, glycine residues that satisfy the above points could be good target residues for site-directed mutagenesis for stabilizing enzymes.

The fact that stability-increasing mutants were obtained by random mutagenesis from a monomeric enzyme of xylanase as well as from a tetrameric glucose dehydrogenase [1] indicates that these enzymes are not optimized in nature for their stability. As this seems to be a common feature of enzymes from mesophiles, there should be much room for stabilizing enzymes by ran-

dom mutagenesis. In addition, this method does not require a knowledge of the tertiary structure of a target enzyme. Thus, random mutagenesis is a good, practical, and generally applicable method for improving the stability and other properties of enzymes.

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Protein Science

Mutational and crystallographic analyses of the active site residues of the Bacillus circulans xylanase

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Mutational and crystallographic analyses of the active site residues of the *Bacillus circulans* xylanase

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Abstract

Using site-directed mutagenesis we have investigated the catalytic residues in a xylanase from *Bacillus circulans*. Analysis of the mutants E78D and E172D indicated that mutations in these conserved residues do not grossly alter the structure of the enzyme and that these residues participate in the catalytic mechanism. We have now determined the crystal structure of an enzyme–substrate complex to 1.8 Å resolution using a catalytically incompetent mutant (E172C). In addition to the catalytic residues, Glu 78 and Glu 172, we have identified 2 tyrosine residues, Tyr 69 and Tyr 80, which likely function in substrate binding, and an arginine residue, Arg 112, which plays an important role in the active site of this enzyme. On the basis of our work we would propose that Glu 78 is the nucleophile and that Glu 172 is the acid-base catalyst in the reaction.

Keywords: catalytic residues; enzyme mechanism; enzyme-substrate complex; nucleophile; xylanase

Glycosidases use a reaction mechanism based on acid catalysis by acidic amino acid residues (Sinnott, 1990). These residues have been identified and probed by X-ray crystallography, protein chemistry, enzymology, and site-directed mutagenesis to determine their roles in catalysis (Phillips, 1967; Rouvinen et al., 1990; Chauvaux et al., 1992; Juy et al., 1992; Mooser, 1992; Davies et al., 1993). These techniques are now being applied to cellulases and xylanases, which already have commercial applications (Yang et al., 1992; Gilbert & Hazlewood, 1993; Sung et al., 1993). As part of the growing interest in protein engineering of enzymes, which can be exploited in industrial processes, endo-β-1,4-D-xylanases (EC 3.2.1.8) have been found to be an effective biochemical means of decreasing the amount of chemical agents required to bleach pulp used in paper production (Viikari et al., 1986; Paice et al., 1992).

We have examined the properties of the *Bacillus circulans* 20-kDa xylanase, which is a member of family G xylanases according to the glycosidase classification scheme of Gilkes et al. (1991), with the goal of engineering it for wider application in the pulp and paper industry. The catalytic mechanism of this enzyme has been probed to understand its substrate specificity and the contributions that specific residues make in determining the activity of this class of enzyme.

Chemical modification studies with another family G xylanase, the *Schizophyllum commune* xylanase (*xyn*A), indicated that acidic residues were involved in catalysis (Bray & Clarke, 1990). These investigators showed the pK_a value for one of these residues was elevated and suggested that the residue was an acid catalyst similar to Glu 35 of hen egg white lysozyme (HEWL). An investigation of the stereochemical course of the reaction with *B. circulans* and *S. commune* xylanases indicated that the reaction proceeds with retention of configuration at the anomeric center (Gebler et al., 1992). The *B. circulans* xylanase also catalyzes transglycosylation (Wakarchuk, unpubl.). These results indicated that the enzyme mechanism is one of general acid catalysis using a nucleophile (or charge-stabilizing residue) and an acid-base catalyst similar to the mechanism of other glycosidases that show retention of the substrate's anomeric configuration in their products (Fig. 1; Sinnott, 1990; Kempton & Withers, 1992; Mooser, 1992). An analysis of a primary sequence alignment of 15 family G xylanases indicated that only 2 glutamic acid residues were absolutely conserved in this family of xylanases (Fig. 2). We, as well as others, have published preliminary data about mutations at these glutamic acid residues (Katsube et al., 1990; Wakarchuk et al., 1992), and we have proposed that Glu 78 and Glu 172 are the catalytic residues. A recent report from Ko et al. (1992) showed that 2 glutamic acid residues were involved in the catalytic mechanism of the *Bacillus pumilus* family G xylanase.

Our mutational analysis has been combined with an X-ray crystallographic structure of an enzyme–substrate complex to provide a more detailed description of the active site and the mechanism of action for this enzyme. We show here that the 2 glutamic acid residues Glu 78 and Glu 172 are intimately involved in catalysis, that Arg 112 also plays a role in cataly-

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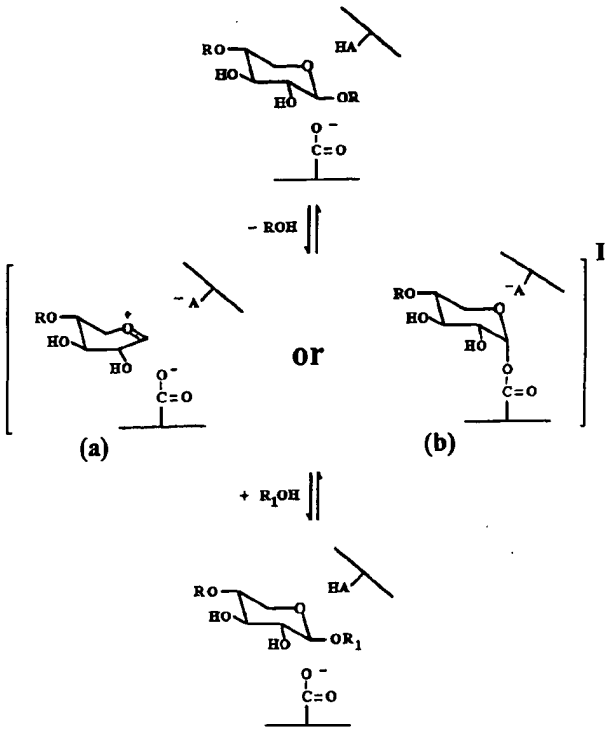

Fig. 1. General reaction mechanism for a retaining endo-β-D-xylanase. The structures of the substrate, intermediates, and product have been simplified for clarity. R = $xyl_{(n)}$, HA = the acid catalyst, I = the structures inside the brackets are possible intermediates, and R_1 = H or $xyl_{(n)}$. The reaction proceeds by a double displacement mechanism where general acid catalysis/nucleophilic attack on the anomeric center produces 1 of 2 possible intermediates. Intermediate (a) has a carboxylate acting in an ion pair to stabilize an oxo-carbonium ion, whereas intermediate (b) shows that the transition state has collapsed into a covalent glycosyl-enzyme. Either of these intermediates could then react with a nucleophilic water (R_1 = H) to produce hydrolysis products or another xylo-oligosaccharide (R_1 = $xyl_{(n)}$) to produce trans-glycosylation products. The transition states for both steps are likely to have substantial oxo-carbonium ion character.

sis, and that Tyr 69 and Tyr 80 are likely involved in substrate binding.

Results

Mutagenesis of conserved amino acid residues

Prior to the solution of the structure of the *B. circulans* xylanase by crystallography, we had used site-directed mutagenesis to examine several acidic amino acid residues that are conserved in the family G xylanases (Fig. 2). Two glutamic acid residues, Glu 78 and Glu 172 (numbering refers to the position in the *B. circulans* sequence), are absolutely conserved, along with 1 additional acidic residue, Asp 83, which is an aspartic acid in the bacterial xylanase sequences and a glutamic acid in the fungal xylanase sequences. Originally, 2 other aspartic acid residues were also targeted for mutagenesis on the basis of their sequence conservation in this family. However, there are now members known in this family that lack these aspartic acid residues, and their overall conservation is 11 of 15 for Asp 11 and 13 of 15 for Asp 106 in these sequences. Once the crystal structure of the *B. circulans* enzyme was known, the absolutely conserved glutamic acid residues were observed in the active site cleft (Campbell et al., 1993).

The specific activities of the acidic residue mutants show that only mutations at Asp 11, Glu 78, and Glu 172 have a significant effect on catalysis (Table 1). The retention of enzyme activity in the D11N mutant shows the acidic side chain of Asp 11 is not essential for catalysis in this enzyme. When the glutamic acid residues are mutated to glutamine (E78Q and E172Q), no enzyme activity can be detected; however, when the carboxylate side chain is maintained but the side-chain length is shortened in E78D and E172D, some residual activity is observed (Table 1). The large decrease in activity seen with the mutant enzymes E78D and E172D, 0.04% and 0.16% residual activity respectively, strongly suggests that these are the catalytic amino acid residues. These residues correspond to the *B. pumilis* xylanase residues Glu 93 and Glu 182, which were shown to be important in that enzyme (Ko et al., 1992).

Table 1. *Enzymatic properties of various xylanase mutants*

Enzyme	Activity (% of wild type)	$K_{m(app)}$ (mg/mL)	$k_{cat(app)}$ (min^{-1})	$k_{cat(app)}/K_{m(app)}$ (min^{-1} mg^{-1})
BCX (wild type)	100	1.9 ± 0.2[a]	8,918	4,693
D11N	25	0.92 ± 0.02	2,245	2,440
E78D	0.043	2.3 ± 0.2	3.9	1.7
E78Q	<0.01[b]	nd[c]	nd	nd
E172D	0.19	4.4 ± 0.5	17.1	3.9
E172Q	<0.01	nd	nd	nd
E172C	<0.01	nd	nd	nd
Y69F	<0.01	nd	nd	nd
Y80F	0.045	12 ± 2	3.7	0.3
Y166F	76	1.2 ± 0.12	6,807	5,672
R112K	83	5.9 ± 0.4	7,373	1,249
R112N	35	5.5 ± 0.7	3,122	568

[a] The confidence levels are the averages of the standard deviation as calculated by Enzfitter.
[b] This is the detection limit of the assay.
[c] Not determined.

Fig. 2. Amino acid sequence alignment of low molecular mass xylanases. The sequence alignment is based on structural considerations, not simply on computer-generated alignments. The sequences of *Bacillus subtilis* and *Trichoderma viride* have been omitted because the *B. subtilis* sequence is identical to *Bacillus circulans* except at position 147, and *T. viride* is essentially identical to *Trichoderma reesei* except at positions 9, 143, 144 (Oku et al., 1993). The sequences are B. pum. = *Bacillus pumilis* (Fukasaki et al., 1984); C. aceto. = *Clostridium acetobutylicum* (Zappe et al., 1990); R. flav. = *Ruminococcus flavefaciens* (Zhang J, Flint HJ, 1992, EMBL Database accession #Z11127); T. ree. II = *T. reesi* XYN II (Torronen et al., 1992); T. harz. = *Trichoderma harzianum* (Yaguchi et al., 1992); S. comm. = *Schizophyllum commune* (Oku et al., 1993); S. sp 36a = *Streptomyces* sp. 36a (Nagashima et al., 1989); S. liv. B = *Streptomyces lividans* XYN B (Shareck et al., 1991); S. liv. C = *S. lividans* XYN C (Shareck et al., 1991); B. circ. = *B. circulans* (Yang et al., 1988); A. niger = *Aspergillus niger* (de Graaff et al., 1992); A. tubig = *Aspergillus tubigenesis* (Maat et al., 1992); T. ree. I = *T. reesei* XYN I (Torronen et al., 1992).

The solution of the high resolution X-ray crystal structure revealed a long, deep cleft formed at the intersection of 2 β-sheets and bordered by an extended, flexible loop (Kinemage 1). The conserved glutamic acid residues were found on either side of the cleft in positions similar to Glu 35 and Asp 52 of HEWL. In addition to the conserved acidic residues, there are a number of other conserved residues that the crystal structure shows are in the active site cleft (Fig. 3). We targeted Tyr 69, Tyr 80, Tyr 166, and Arg 112 to investigate the role that these residues may play in the reaction mechanism. Mutation of the conserved Tyr 80 to phenylalanine (Y80F) resulted in a dramatic loss of activity, leaving only 0.03% residual activity (Table 1), whereas the mutation Y69F resulted in a total loss of detectable enzyme activity. In contrast to these other tyrosine mutants, the mutation Y166F has only a minor effect on enzyme function. The Tyr 166 residue is not absolutely conserved in this enzyme family (Fig. 2), and consequently the contribution of the hydroxyl group may not be that important. Mutation of the conserved Arg 112 to lysine (R112K) also resulted in no change in the enzyme activity, but conversion to asparagine (R112N) resulted in a 68% drop in specific activity. Those mutants showing a decreased but measurable enzymatic activity were examined further for their kinetic properties.

Kinetic analyses of the active site mutants

The kinetic analysis of xylanases is hampered by the lack of a suitable synthetic substrate for colorimetric measurements. Therefore the analysis of the active site mutants had to be performed using a reducing sugar assay to measure hydrolysis of a soluble xylan preparation. There has been much discussion in the literature about the merits of various reducing sugar assays, but we chose to use the assay reagent described by Lever (1972). This assay has very sensitive detection limits (μM) and is insensitive to the chain length of the released oligosaccharides (data not shown), which allows a more accurate estimation of the newly generated reducing ends. The major limitation of the assay method is that the inhomogeneous substrate changes during the reaction ($K_{m(app)}$ increases with time, data not shown), so particular care was taken to ensure that the extent of hydro-

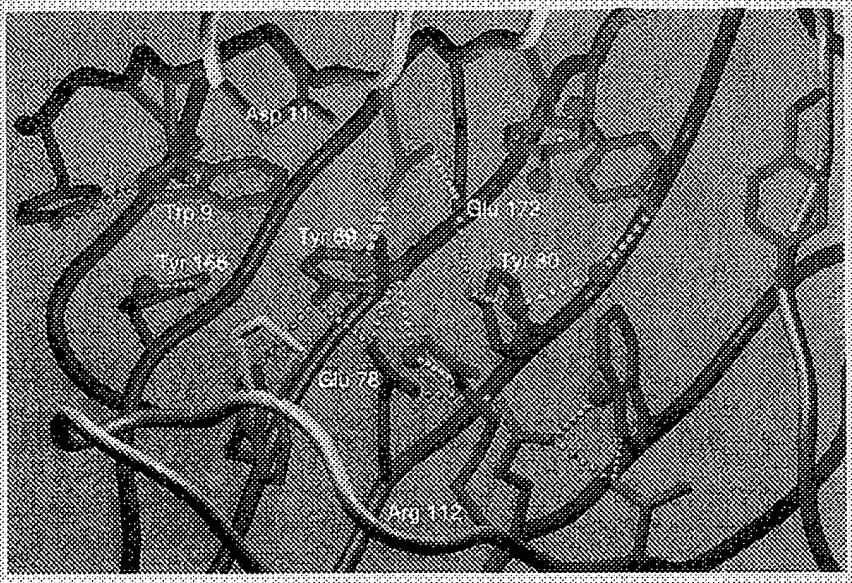

Fig. 3. The active site cleft of the *B. circulans* xylanase. The structure of the enzyme has been presented here with the molecular graphics program SETOR (Evans, 1993), with the carbon backbone shown as a gold tube and selected side chains displayed within the active site cleft. The residues are colored according to residue type, with Glu and Asp in red, Tyr in green, Trp in magenta, Arg in blue and Gln and Asn in cyan. Potential hydrogen bonds were calculated using the default criteria in the program QUANTA (Molecular Simulations, Inc.) and are shown here as the yellow dotted lines. The blue spheres are water molecules.

lysis was the same for each enzyme examined, such that a relative comparison of kinetic parameters would be possible.

Despite the limitation of our reducing sugar assay, it was sensitive enough to allow analysis of the E78D, E172D, and Y80F mutant proteins, which had very low levels of enzyme activity (Table 1). There was only a minor increase in the $K_{m(app)}$ for the E172D enzyme and no change in the $K_{m(app)}$ for the E78D enzyme. This indicates that the active site structure of the enzyme must be maintained in these mutants and that the major effect of these mutations is on catalysis rather than substrate binding. In contrast to the other mutants, the Y80F enzyme showed a moderate increase in the $K_{m(app)}$ for the soluble xylan substrate and a large decrease in catalytic activity.

Withers et al. (1992) have shown that 2 of their β-glucosidase active site mutants, E358N and E358Q, had small amounts of contaminating activity that could be attributed to either the corresponding Asp mutant or wild type. We considered the possibility that the low level of activity observed in some mutants was from a small amount of contaminating wild-type enzyme produced by translational misreading in *Escherichia coli* or spontaneous deamidation of the amide mutants; however, we never observed residual activity in enzyme preparations from the mutants E78Q, E172Q, E172C, and Y69F. Also in the case of the Y80F mutant, the $K_{m(app)}$ is significantly higher than the wild type. Therefore, we believe that the observed activity is from the mutant proteins.

CD spectra of the active site mutants

To insure the mutant proteins were not improperly folded, we analyzed the active site mutants by CD spectroscopy. The spectra are basically unremarkable, with the E78D and Y80F mutants showing a slight deepening of the trough at 220 nm (Fig. 4). All of the other mutants analyzed show spectra that overlap with the wild type. These data suggest that the reduced activity of these mutants as well as the inactive mutants (e.g., Y69F) is not due to any gross alterations in the structure.

Structure of an enzyme–substrate complex

The solution of the high resolution X-ray crystal structure of the *B. circulans* xylanase allowed identification of the conserved residues in the active site of the enzyme (Fig. 3; Kinemage 2; Campbell et al., 1993). The presence of several tyrosines in the cleft suggests likely sites for the formation of hydrogen bonds to the substrate. It was, however, not obvious how the substrate would

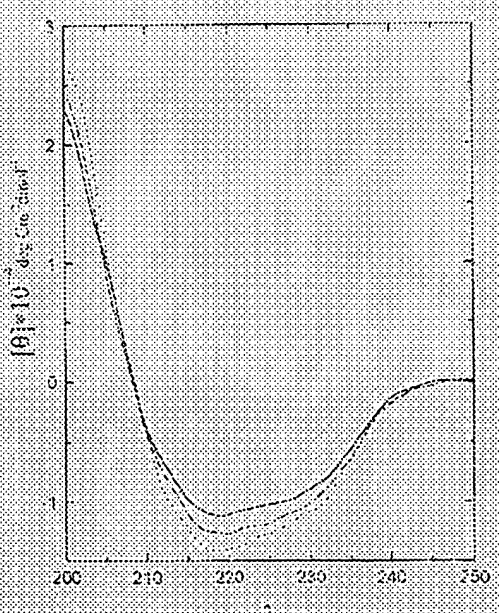

Fig. 4. CD spectra of selected active site mutants of the *B. circulans* xylanase. The wild-type enzyme spectrum is shown by the solid line, the E78D mutant spectrum is shown by the – – – line, and the Y80F spectrum is shown by the mixed ——— line.

fit into the cleft of the enzyme. We attempted to produce co-crystals of the enzyme and small xylo-oligosaccharide substrates using the catalytically incompetent mutant E78Q, without success. Previously we had prepared several single cysteine mutants for making heavy atom derivatives for crystallography. One of those, E172C, was not enzymatically active (Table 1) and we succeeded in producing co-crystals of this mutant with xylotetraose.

The structure of the enzyme is essentially unchanged upon binding the substrate. The 2 structures superimpose with an RMS deviation of 0.137 Å for all main-chain atoms. The flexible loop (residues 111–131) moves only a small amount, opening the cleft to accommodate the substrate. The Cα atoms of residues 118–120 shift by between 0.6 and 0.7 Å. The only other changes in the protein are at the site of the mutation and a rotation of the side chain of Asn 35, which forms a hydrogen bond to Glu 172 in the native structure.

Only 2 xylose residues could be fitted into the electron density, suggesting that either the enzyme has a very small amount of residual activity in the crystal or that the enzyme requires a larger substrate for tight binding. The electron density that is observed for the substrate is weaker than for the nearby protein atoms including the flexible loop region, suggesting that the substrate is bound at less than 100% occupancy. The structure was initially refined with the occupancy set to 100% and the average *B*-factor was found to be 26 Å^2 for the substrate, whereas the average *B*-factor for the main-chain atoms of the loop region was 16 Å^2. Refinement of the *B*-factors with the occupancy set to 70% resulted in the average *B*-factor for the substrate of 16 Å^2. When the difference electron density map was contoured at a high level (4σ), density was observed primarily at the positions of the oxygen atoms (data not shown), thus unambiguously demonstrating the direction of the carbohydrate chain. The refined xylobiose structure had ϕ and ψ angles of 120° and 163°, respectively, similar to the ideal geometry of 175° and 135°, respectively. When the difference electron density map was contoured at 2.5σ, a small tail of density was seen extending beyond the O4 oxygen at the nonreducing end of the first visible xylose residue (Fig. 5). This suggests that the rest of the substrate extends beyond the active site cleft and is too mobile to be seen in the electron density.

The structure of this complex revealed some of the enzyme–substrate interactions (Fig. 6; Kinemages 2, 3). A stacking interaction of Trp 9 with one of the xylose rings is evident as are hydrogen bonds between the 2 xylose rings and Tyr 69, Tyr 166, Arg 112, and the backbone carbonyl of Pro 116 (Table 2). The terminal oxygen of the reducing end of the xylotetraose is in close proximity to the 2 glutamic acid residues.

Table 2. *Summary of close contacts between B. circulans xylanase and the substrate xylotetraose*

Substrate ring	Substrate atom	Protein atom	Distance (Å)
Xyl 1	O3	OH Tyr 166	2.82
	O2	OH Tyr 69	2.88
	C5	CE2 Trp 9	3.37
	C5	NE1 Trp 9	3.10
Xyl 2	C1	OE1 Glu 78	3.37
	O1	OE1 Glu 172[a]	2.15
	O2	OE2 Glu 78	3.00
	C1	OH Tyr 80	3.57
	O2	NE Arg 112	2.97
	O3	NH2 Arg 112	3.15
	O3	O Pro 116	2.56

[a] Position of Glu 172 is taken from the native structure after superimposition onto the complex structure.

Discussion

The reaction mechanism of retaining β-glycosidases has been examined for many years, and it was first determined in HEWL that 2 acidic residues were involved in catalysis (Blake et al., 1967; Phillips, 1967). Several other glycosidases have now been examined and the lysozyme paradigm for acid catalysis has been a common theme among them (Sinnott, 1990; Mooser, 1992). An extension of these earlier observations is possible when one can assign a role for a specific residue within the active site. Considering the proposed mechanism for retaining glycosidases, it is important to determine which residue is the nucleophile (or charge-stabilizing residue) and which is the acid-base catalyst. The nucleophile involved in a β-glucosidase, a β-galactosidase,

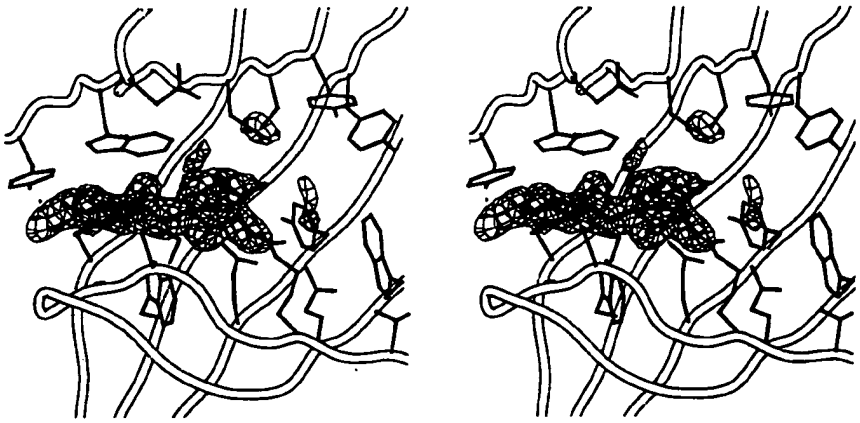

Fig. 5. Stereo diagram of the $F_o - F_c$ difference map for the E172C-xylotetraose complex. The phases were calculated from the refined structure and F_c was calculated after omitting the substrate and all of the water molecules.

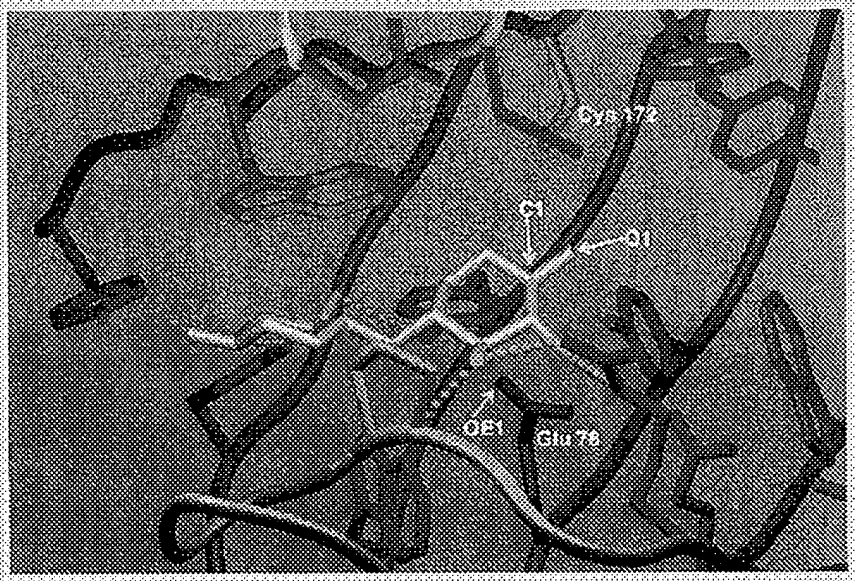

Fig. 6. View of the *B. circulans* xylanase active site for the mutant E172C, in complex with xylotetraose. This representation is similar to that shown in Figure 2, except that Cys 172 is shown in gold, and some residue labels have been omitted for clarity. Atoms C1 and O1 of the second xylose residue are indicated. Only potential hydrogen bonds to the substrate have been shown.

a cellobiohydrolase, and an endoglucanase have been trapped through the use of suicide inhibitors (Withers & Street, 1988; Gebler et al., 1991; Tull et al., 1991; Wang et al., 1993). The only published high resolution structures for retaining endo-β-glycosidases are lysozymes. The crystallographic assignment of Asp 52 as the charge-stabilizing residue in HEWL comes from the early work of Blake et al. (1967) and from the more recent examination of the structure by Strynadka and James (1991). The Asp 20 residue in T4 phage lysozyme is thought to play a similar role to Asp 52 in HEWL (Anderson et al., 1981). In our enzyme-substrate complex, the orientation of the substrate suggests that Glu 78 and Glu 172 would be in position to act as the nucleophile and the acid–base catalyst, respectively. The distance from the OE1 atom of E78 to the C1 atom of the reducing end of the xylotetraose (Fig. 6; Table 2) is similar to the distance from OD2 of Asp 52 to C1 of the saccharide in subsite D from a trisaccharide complex with HEWL (Strynadka & James, 1991). Supporting evidence for the role of Glu 78 as the nucleophile is the proximity of Arg 112 to Glu 78. Although no salt link is found in this structure, unlike the situation in the *B. pumilis* xylanase (Katsube et al., 1990), the presence of a positive charge in the vicinity may help to lower the pK_a of Glu 78, thus maintaining it in a negatively charged state. In addition, mutational analysis shows that enzyme activity is more affected in the E78D mutant than in the E172D mutant, which is consistent with E78 having the more critical role of the nucleophile.

The Glu 172 position relative to the xylotetraose can be calculated by superimposition of the native structure onto that of the complex (Table 2). From this structure it can be seen that the positioning of Glu 172 relative to the hydroxyl group at C1 (O1) of xyl 2 is similar to that of Glu 35 in HEWL relative to the hydroxyl group at C1 of the NAM-NAG-NAM substrate (Strynadka & James, 1991). The distance between OE2 of Glu 172 and O1 of xyl 2 is shorter than a normal hydrogen bond. Therefore, in order to fit this substrate into the cleft of the native enzyme, further movement of the flexible loop would be required. Attempts to model substrates into the active site were prevented by steric hindrance with residues in the flexible loop (data not shown). Glutamic acid residues 78 and 172 are exposed to solvent to the same degree and both side-chain oxygens of each are within hydrogen bonding distance of other residues in the active site. In the native enzyme structure Glu 78 is hydrogen bonded to Gln 127 and Tyr 69, whereas Glu 172 is hydrogen bonded to Asn 35 and Tyr 80 (Fig. 3). It should be noted that this structure represents only a partial view of the enzyme-substrate interactions. A larger substrate spanning the whole active site may bind in a slightly different conformation likely with an accompanying change in the position of the loop. This would change the distances from the potential nucleophile and acid-base catalyst to the saccharide.

The structures of several carbohydrate-binding proteins and glycosidases have been determined (Rouvinen et al., 1990; Vyas, 1991; Juy et al., 1992; Mooser, 1992; Davies et al., 1993; Keitel et al., 1993). There are many features of carbohydrate-binding proteins and glycosidases that are similar, though the structures of these binding sites or active sites are quite different. The most striking similarity is the large number of aromatic residues, mainly tyrosine and tryptophan, which comprise the binding or active sites. The *B. circulans* active site contains 6 tyrosine and 3 tryptophan residues. The stacking interaction of Trp 9 with the face of a xylose ring is similar to a predicted stacking interaction of Trp 457, with a glucose residue at subsite D in the CelD active site (Juy et al., 1992), and also similar to the observed stacking interactions of Trp 135 with the face of a glucose residue in subsite A and that of Trp 367 with the face of a glucose residue in subsite C of the CBHII active site (Rouvinen et al., 1990). HEWL was the first retaining glycosidase for which this kind of aromatic stacking interaction with a sugar face was seen in the crystallographically determined protein structure (Phillips, 1967). There is a stacking interaction between Trp 62 and the nonpolar face of the *N*-acetylmuramic acid ring in subsite B (Strynadka & James, 1991). This stacking interaction appears to be a very common binding interaction in glycosidases.

The role of the tyrosines in the active site of *B. circulans* xylanase is less certain than the role played by the Trp 9 residue. Tyrosine residues have been implicated in the catalytic mecha-

nism for both the *E. coli* β-galactosidase (Mooser, 1992) and the *Agrobacterium faecalis* β-glucosidase (Withers, pers. comm.). In both cases the mutation of a single tyrosine to phenylalanine resulted in a dramatic decrease in activity. In the case of the *E. coli* β-galactosidase, it is hypothesized that Tyr 503 may be the proton donor in the reaction. In the β-glucosidase it is possible that Tyr 298 has a hydrogen bonding role with the C2-hydroxyl of the substrate. In the structure of the *B. circulans* xylanase complexed with xylotetraose, hydroxyl groups of the xylose rings are close enough to form hydrogen bonds with the hydroxyl groups of Tyr 69 and Tyr 166 (Table 2). Why then does the mutation Y69F completely inactivate the protein, whereas the mutant Y166F has only a negligible effect on enzyme activity? From the structure of the complex it can be calculated that Tyr 69 makes 2 hydrogen bonds, one with the C2 OH group of the nonreducing (second) xylose ring, and the second with the OE1 atom of Glu 78. This hydrogen bond to Glu 78 may help orient the nucleophile for the reaction, whereas the second helps to position the substrate. For enzyme activity then, the single hydrogen bond from Tyr 166 to the C3 OH of the second xylose ring appears to be less important for the reaction than those from Tyr 69.

Kinetic analysis of the Y80F mutant enzyme suggests a role for Tyr 80 in substrate binding. The closest atom of xyl 2 to the hydroxyl of Tyr 80 is C1 (Table 2; Fig. 6). This distance is slightly longer than that for Glu 78 to C1, suggesting that Tyr 80 does not have a direct role in catalysis. The steric conflicts seen in modeling the substrate as discussed previously would imply that the Tyr 80–C1 distance must be even longer in the complex of the substrate with the wild-type enzyme. In a complex with a longer substrate, Tyr 80 may have a role in hydrogen bonding to the substrate. In the native structure it appears to make a hydrogen bond to Glu 172 and thus may have a role in positioning the proposed acid–base catalyst.

In summary then, it would appear that disruption of the hydrogen bonds from the active site tyrosine residues to the nucleophile are more detrimental to enzyme activity than disruption of the hydrogen bonds to either the acid catalyst or the substrate alone. Experiments to examine the substrate binding interactions of this enzyme further and to produce a better complex that may answer some of these questions are under way.

Materials and methods

Bacterial strains, growth conditions, and standard methods

The following standard laboratory strains of *E. coli* were used for the propagation of recombinant plasmids, and the production of recombinant gene products: MV1190: Δ(*lac-proAB*), *thi*, *supE*44, Δ(*sr*1-*recA*)306::Tn10(tet^r) [F′: *traD*36, *proAB*, *lacI*^{q} ΔM15]; RZ1032: HfrKL16 PO/45 [*lysA*(61–62)], *dut*1, *ung*1, *thi*1, *relA*1, *Zbd*-279::Tn10, *supE*44; BHM 71-18: Δ(*lac-proAB*), *thi*, *supE*44, [*mut*S::Tn10(tet^r)], [F′: *proAB*, *lacI*^{q} ΔM15]; HB101: *hsdS*20 (r− m−), *leu*, *supE*44, *ara*14, *galK*2, *lacY*1, *proA*2, *rpsL*20 (Str^r), *xyl*-5, *mtl*-5, *recA*13, *mcrB*.

The synthetic gene for the *B. circulans* protein has been described (Sung et al., 1993). The gene was transferred to the vector used in this work, pCWori+. This was obtained from Ms. Amy Roth of the University of Oregon and it is a 5-kb pBR322 derivative, containing the ampicillin resistance marker, the *lacI* gene, part of the *lacZ* structural gene, 2 copies of the synthetic TAC promoter, and 1 copy of the lacUV5 promoter; in addition it carries the M13 phage origin of replication, which confers the ability to produce single-stranded DNA (ssDNA) when cells containing it are superinfected with a helper phage. All liquid cultures were grown in either 2YT medium (16 g yeast extract, 10 g bacto-tryptone, 5 g NaCl, 1 L of H_2O), or TB medium (24 g yeast extract, 12 g bacto-tryptone, 10 mL 1 M potassium phosphate buffer, pH 7.5, 5 mL of 80% glycerol, 1 L H_2O). The antibiotic ampicillin was added at 150 mg/L to all cultures of plasmid-containing strains. The cultures were grown with shaking at 30 °C for protein and plasmid production, and at 37 °C for the production of ssDNA-containing particles.

Basic recombinant DNA methods such as plasmid DNA isolation, restriction enzyme digestions, the purification of DNA fragments for cloning, ligations, transformations, and DNA sequencing were performed as recommended by the enzyme supplier or the manufacturer of the kit used for the particular procedure. Restriction and DNA modification enzymes were purchased from New England Biolabs Ltd., Mississauga, Ontario. Prep-A-Gene DNA purification matrix was purchased from Bio-Rad laboratories, Mississauga, Ontario, Canada. Sequenase, DNA sequencing kit was purchased from US Biochemicals, Cleveland, Ohio. Oligonucleotide 3′ end labeling with digoxigenin–ddUTP and the subsequent chemiluminescent detection were performed with a kit and some additional reagents from Boehringer Mannheim Canada, Laval, Quebec. Protein concentration was determined from the molar extinction coefficient of the xylanase: 81,790 L mol^{-1}, $A_{280}^{0.1\%} = 4.08$; no correction was made for the Tyr to Phe mutations.

Site-directed mutagenesis

Mutagenesis was performed either using the UDNA method as described by Kunkel et al. (1987) or by constructing synthetic restriction fragment cassettes for replacement of specific regions of the gene (Sung et al., 1993). Oligonucleotides were synthesized using an Applied Biosystems model 380A DNA synthesizer, using the phosphoramidite method. Synthetic oligonucleotides were purified by polyacrylamide gel electrophoresis in 15% gels containing 7 M urea. The clones containing a functional xylanase gene were identified by screening transformants on plates containing remazol-brilliant blue xylan. Colonies that expressed the gene for an active xylanase were identified by the production of halos (Kluepfel, 1988). Inactive mutants were identified from colony hybridization reactions with the mutagenic oligonucleotide as a probe. Clones with a positive hybridization signal were sequenced and then repurified by transformation to ensure the purity of the clone. DNA sequencing was again performed to verify that there were no other changes other than at the desired codons.

Protein purification was performed as described previously (Sung et al., 1993), with the modification that the ion exchange column was a POROS HSII perfusion column (Perseptive Biosystems, Inc.).

Measurement of enzymatic activity

The activity of the enzyme was measured by the quantitative assay of the number of reducing sugar end groups generated from hydrolysis of soluble xylan. The substrate for this assay was the

fraction of birchwood xylan that dissolved in water from a 5% suspension of birchwood xylan (Sigma Chemical Company). After removing the insoluble fraction, the supernatant was freeze dried and stored in a desiccator.

The measurement of specific activity was performed as follows. Reaction mixtures contained 10 mg/mL xylan in assay buffer (20 mM MES-NaOH, 50 mM NaCl, pH 6.0), with enzyme suitably diluted in 1 mg/mL bovine serum albumin, in assay buffer. The substrate and buffer were mixed and prewarmed to 40 °C, and the reaction was started by the addition of the enzyme. At various time intervals 50-μL portions were removed and the reaction was stopped by dilution into 1 mL of 5 mM NaOH. The amount of reducing sugars was determined with the hydroxybenzoic acid hydrazide reagent (Lever, 1972). A unit of enzyme activity was defined as that amount generating 1 μmol reducing sugar in 1 min at 40 °C, using xylose as a standard.

For the determination of kinetic parameters, substrate concentrations from 0.4 mg/mL to 20 mg/mL were used. The time used for the enzyme reactions was 5 min, using a final protein concentration of 300 ng/mL for the wild type. Mutant proteins were used at concentrations that gave the same overall hydrolysis level as the wild-type control. Kinetic parameters were calculated using the computer program Enzfitter (Leatherbarrow, 1987).

CD spectroscopy

The CD spectra of the xylanase proteins was obtained from a JASCO J-600 spectropolarimeter. The instrument was calibrated with ammonium-*d*-camphorsulfonate. Spectra in the far UV region (200–250 nm) were measured in a 0.02-cm-pathlength cylindrical quartz cell at a protein concentration of 0.5 mg/mL.

X-ray crystallography

Crystals of the complex of the *B. circulans* xylanase mutant E172C and xylotetraose were grown by the hanging drop vapor diffusion method. The reservoir buffer was 40 mM Tris, pH 7.5, 22% saturated $(NH_4)_2SO_4$, and 100 mM NaCl. The initial protein concentration in the droplet was about 4 mg/mL after adding an equal volume of reservoir solution to the protein solution with a 10-fold molar excess of xylotetraose. Droplets were seeded after 1 day of equilibration. The space group of the crystals was $P2_12_12_1$ with $a = 44.00$ Å, $b = 52.78$ Å, and $c = 78.39$ Å. The X-ray diffraction data were collected on a San Diego Multiwire Area Detector system on a Rigaku rotating anode generator. All data reduction was performed using the San Diego software and the PHASES program package (Furey & Swaminathan, 1990). The initial electron density map was calculated with phases from the native protein structure (Campbell et al., 1993), which has been refined to an *R*-factor of 0.165 for data between 8 and 1.49 Å resolution. The starting conformation of the xylotetraose substrate was an idealized structure provided by Dr. David Bundle. The values for the torsion angles, ϕ (measured from atoms C2-C1-O4-C4) and ψ (C1-O4-C4-C3) were 175° and 135°, respectively, in the ideal model. The substrate was built into the difference electron density with O (Jones et al., 1990) and the complete structure (with the occupancy of the substrate maintained at 100%) was refined using the simulated annealing and minimization protocols of X-PLOR (Brünger, 1988). The final model contains 1,445 non-hydrogen protein atoms, 2 xylose residues, 1 sulfate molecule, and 144 water molecules. The resulting *R*-factor was 0.161 for data between 8 and 1.8 Å resolution with $F > 2\sigma(F)$. RMS deviations from ideality are 0.008 Å for bonds, 1.66° for angles, 27.28° for dihedrals, and 1.26° for impropers.

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DOCUMENT TYPE: Journal
LANGUAGE: English
AB Wheat endoxylanase inhibitor TAXI-I inhibits microbial glycoside hydrolase **family 11** endoxylanases. Crystallog. data of an Aspergillus niger endoxylanase-TAXI-I complex showed His374 of TAXI-I to be a key residue in endoxylanase inhibition. Its role in enzyme-inhibitor interaction was further investigated by site-directed mutagenesis of His374 into alanine, glutamine or lysine. Binding kinetics and affinities of the mol. interactions between A. niger, Bacillus subtilis, Trichoderma longibrachiatum endoxylanases and wild-type TAXI-I and TAXI-I His374 **mutants** were determined by surface plasmon resonance anal. Enzyme-inhibitor binding was in accordance with a simple 1: 1 binding model. Association and dissociation rate consts. of wild-type TAXI-I towards the endoxylanases were in the range between 1.96 and 36.1 + 104 M-1·s-1 and 0.72-3.60 + 10-4·s-1, resp., resulting in equilibrium dissociation consts. in the low nanomolar range. Mutation of TAXI-I His374 to a variable degree reduced the inhibition capacity of the inhibitor mainly due to higher complex dissociation rate consts. (three- to 80-fold increase). The association rate consts. were affected to a smaller extent (up to eightfold decrease). **Substitution** of TAXI-I His374 therefore strongly affects the affinity of the inhibitor for the enzymes. In addition, the results show that His374 plays a critical role in the stabilization of the endoxylanase-TAXI-I complex rather than in the docking of inhibitor onto enzyme.
REFERENCE COUNT: 29 THERE ARE 29 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 2 OF 16 CAPLUS COPYRIGHT 2006 ACS on STN
ACCESSION NUMBER: 2005:999905 CAPLUS
TITLE: A single amino acid **substitution** enhances the catalytic activity of **family 11 xylanase** at alkaline pH
AUTHOR(S): Shibuya, Hajime; Kaneko, Satoshi; Hayashi, Kiyoshi
CORPORATE SOURCE: Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, 305-8687, Japan
SOURCE: Bioscience, Biotechnology, and Biochemistry (2005), 69(8), 1492-1497
CODEN: BBBIEJ; ISSN: 0916-8451
PUBLISHER: Japan Society for Bioscience, Biotechnology, and Agrochemistry
DOCUMENT TYPE: Journal
LANGUAGE: English

AB Random mutagenesis of the gene encoding **family 11** **xylanase** was used to obtain alkalophilic **mutants**. The catalytic domain of the chimeric enzyme Stx15, which was constructed from Streptomyces lividans **xylanase** B and Thermobifida fusca **xylanase** A, was mutated using error-prone PCR and screened for halo formation on dye-linked xylan plates and activity toward soluble xylan. A pos. **mutant**, M1011, was isolated, and it was found that mutation A49V was responsible for the alkalophilicity of the **mutant**. Mutation A49V increased the specific activity at pH 9.1 and the stability of **mutant** A49V was not significantly different from that of Stx15 at 60°C. Both enzymes retained more than 90% of their relative activity from pH 4.7 to 9.1 after 1 h of incubation at 60°C. Anal. of the kinetic parameters at various pH values showed that the A49V mutation reduced the Km in the alkaline pH range, resulting in the higher specific activity of the A49V **mutant** enzyme.

REFERENCE COUNT: 21 THERE ARE 21 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 3 OF 16 CAPLUS COPYRIGHT 2006 ACS on STN

ACCESSION NUMBER: 2005:117815 CAPLUS

DOCUMENT NUMBER: 142:311971

TITLE: Improving the alkalophilic performances of the Xyl1 **xylanase** from Streptomyces sp. S38: structural comparison and mutational analysis

AUTHOR(S): de Lemos Esteves, Frederic; Gouders, Thierry; Lamotte-Brasseur, Josette; Rigali, Sebastien; Frere, Jean-Marie

CORPORATE SOURCE: Centre d'Ingenierie des Proteines, Institut de Chimie, B6a, Universite de Liege, Liege, B-4000, Belg.

SOURCE: Protein Science (2005), 14(2), 292-302
CODEN: PRCIEI; ISSN: 0961-8368

PUBLISHER: Cold Spring Harbor Laboratory Press

DOCUMENT TYPE: Journal

LANGUAGE: English

AB Endo-β-1,4- **xylanases** of the **family 11** glycosyl-hydrolases are catalytically active over a wide range of pH. Xyl1 from Streptomyces sp. S38 belongs to this family, and its optimum pH for enzymic activity is 6. Xyn11 from Bacillus agaradhaerens and XylJ from Bacillus sp. 41M-1 share 85% sequence identity and have been described as highly alkalophilic enzymes. In an attempt to better understand the alkalophilic adaptation of **xylanases**, the three-dimensional structures of Xyn11 and Xyl1 were compared. This comparison highlighted an increased number of salt-bridges and the presence of more charged residues in the catalytic cleft as well as an eight-residue-longer loop in the alkalophilic **xylanase** Xyn11. Some of these charges were introduced in the structure of Xyl1 by site-directed mutagenesis with **substitutions** Y16D, S18E, G50R, N92D, A135Q, E139K, and Y186E. Furthermore, the eight addnl. loop residues of Xyn11 were introduced in the homologous loop of Xyl1. In addition, the coding sequence of the XylJ catalytic domain was synthesized by recursive PCR, expressed in a Streptomyces host, purified, and characterized together with the Xyl1 **mutants**. The Y186E **substitution** inactivated Xyl1, but the activity was restored when this mutation was combined with the G50R or S18E **substitutions**. Interestingly, the E139K mutation raised the optimum pH of Xyl1 from 6 to 7.5 but had no effect when combined with the N92D **substitution**. Modeling studies identified the possible formation of an interaction between the introduced lysine and the substrate, which could be eliminated by the formation of a putative salt-bridge in the N92D/E139K **mutant**.

REFERENCE COUNT: 40 THERE ARE 40 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 4 OF 16 CAPLUS COPYRIGHT 2006 ACS on STN
ACCESSION NUMBER: 2004:356690 CAPLUS
DOCUMENT NUMBER: 141:102220
TITLE: Acidophilic adaptation of **family 11** endo-β-1,4- **xylanases**: modeling and mutational analysis
AUTHOR(S): De Lemos Esteves, Frederic; Ruelle, Virginie; Lamotte-Brasseur, Josette; Quinting, Birgit; Frere, Jean-Marie
CORPORATE SOURCE: Centre d'Ingenierie des Proteines, Institut de Chimie, B6a, Universite de Liege, Liege, B-4000, Belg.
SOURCE: Protein Science (2004), 13(5), 1209-1218
CODEN: PRCIEI; ISSN: 0961-8368
PUBLISHER: Cold Spring Harbor Laboratory Press
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Xyl1 from Streptomyces sp. S38 belongs to the low mol. mass **family 11** of endo-β-1,4- **xylanases**. Its three-dimensional structure has been solved at 2.0 Å and its optimum temperature and pH for enzymic activity are 60°C and 6.0, resp. Aspergillus kawachii **xylanase** XynC belongs to the same family but is an acidophilic enzyme with an optimum pH of 2.0. Structural comparison of Xyl1 and XynC showed differences in residues surrounding the two glutamic acid side chains involved in the catalysis that could be responsible for the acidophilic adaptation of XynC. Mutations W20Y, N48D, A134E, and Y193W were introduced by site-directed mutagenesis and combined in multiple **mutants**. Trp 20 and Tyr 193 are involved in substrate binding. The Y193W mutation inactivated Xyl1 whereas W20Y decreased the optimum pH of Xyl1 to 5.0 and slightly increased its specific activity. The N48D mutation also decreased the optimum pH of Xyl1 by one unit. The A134E **substitution** did not induce any change, but when combined with N48D, a synergistic effect was observed with a 1.4 unit decrease in the optimum pH. Modeling showed that the orientations of residue 193 and of the fully conserved Arg 131 are different in acidophilic and "alkaline" **xylanases** whereas the introduced Tyr 20 probably modifies the pKa of the acid-base catalyst via residue Asn 48. Docking of a substrate analog in the catalytic site highlighted striking differences between Xyl1 and XynC in substrate binding. Hydrophobicity calcns. showed a correlation between acidophilic adaptation and a decreased hydrophobicity around the two glutamic acid side chains involved in catalysis.
REFERENCE COUNT: 42 THERE ARE 42 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 5 OF 16 CAPLUS COPYRIGHT 2006 ACS on STN
ACCESSION NUMBER: 2003:434726 CAPLUS
DOCUMENT NUMBER: 139:19034
TITLE: Trichoderma **reesei xylanase** II enzymes with enhanced thermophilicity and alkalophilicity
INVENTOR(S): Sung, Wing L.
PATENT ASSIGNEE(S): National Research Council of Canada, Can.
SOURCE: PCT Int. Appl., 105 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2003046169	A2	20030605	WO 2002-CA1758	20021120
WO 2003046169	A3	20040415		

W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN,

CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW
RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG

CA 2435527	AA	20030605	CA 2002-2435527	20021120
AU 2002342464	A1	20030610	AU 2002-342464	20021120
SE 2003002103	A	20030919	SE 2003-2103	20030718
SE 526842	C2	20051108		
FI 2003001094	A	20030721	FI 2003-1094	20030721
PRIORITY APPLN. INFO.:			US 2001-990874 A	20011121
			WO 2002-CA1758 W	20021120

AB The present invention provides a **xylanase**, or a modified **xylanase** enzyme comprising at least one **substituted** amino acid residue at a position selected from the group consisting of amino acid **11**, **116**, **118**, **144** and **161**, the position determined from sequence alignment of the modified **xylanase** with Trichoderma **reesei xylanase** II amino acid sequence. The **xylanases** described herein exhibit improved thermophilicity, alkalophilicity, expression efficiency, or a combination thereof, in comparison to a corresponding native **xylanase**. The maximum effect temperature is 69-84° and the max effective pH is 5.8-8.4. The improved properties of **xylanase** II muteins are of use in industrial processes, such as paper pulp manufacture

L10 ANSWER 6 OF 16 CAPLUS COPYRIGHT 2006 ACS on STN

ACCESSION NUMBER:	2002:545433 CAPLUS
DOCUMENT NUMBER:	137:244453
TITLE:	Evidence for temporal regulation of the two Pseudomonas cellulosa **xylanases** belonging to glycoside hydrolase **family 11**
AUTHOR(S):	Emami, Kaveh; Nagy, Tibor; Fontes, Carlos M. G. A.; Ferreira, Luis M. A.; Gilbert, Harry J.
CORPORATE SOURCE:	Department of Biological and Nutritional Sciences, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, UK
SOURCE:	Journal of Bacteriology (2002), 184(15), 4124-4133 CODEN: JOBAAY; ISSN: 0021-9193
PUBLISHER:	American Society for Microbiology
DOCUMENT TYPE:	Journal
LANGUAGE:	English

AB P. cellulosa is a highly efficient xylan-degrading bacterium. Genes encoding 5 **xylanases** and several accessory enzymes, which remove the various side chains that decorate the xylan backbone, have been isolated from the pseudomonad and characterized. The **xylanase** genes consist of xyn10A, xyn10B, xyn10C, xyn10D, and xyn11A, which encode Xyn10A, Xyn10B, Xyn10C, Xyn10D, and Xyn11A, resp. In this study a 6th **xylanase** gene, xyn11B, was isolated which encodes a 357-residue modular enzyme, designated Xyn11B, comprising a glycoside hydrolase **family 11** catalytic domain appended to a C-terminal X-14 module, a homolog of which binds to xylan. Localization studies showed that the 2 **xylanases** with glycoside hydrolase family (GH) **11** catalytic modules, Xyn11A and Xyn11B, are secreted into the culture medium, whereas Xyn10C is membrane-bound. xyn10C, Xyn10D, xyn11A, and xyn11B were all abundantly expressed when the bacterium was cultured on xylan or β-glucan but not on medium containing mannan, whereas glucose repressed transcription of these genes. Although all of the **xylanase** genes were induced by the same polysaccharides, temporal regulation of xyn11A and xyn11B was apparent on xylan-containing media.

Transcription of xyn11A occurred earlier than transcription of xyn11B, which is consistent with the predicted mode of action of the encoded enzymes. Xyn11A, but not Xyn11B, exhibits xylan esterase activity, and the removal of acetate side chains is required for **xylanases** to hydrolyze the xylan backbone. A transposon **mutant** of P. cellulosa in which xyn11A and xyn11B were inactive displayed greatly reduced extracellular but normal cell-associated **xylanase** activity, and its growth rate on medium containing xylan was indistinguishable from wild-type P. cellulosa. Based on the data presented here, we propose a model for xylan degradation by P. cellulosa in which the GH11 enzymes convert decorated xylans into **substituted** xylooligosaccharides, which are then hydrolyzed to their constituent sugars by the combined action of cell-associated GH10 **xylanases** and side chain-cleaving enzymes.

REFERENCE COUNT: 33 THERE ARE 33 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 7 OF 16 CAPLUS COPYRIGHT 2006 ACS on STN

ACCESSION NUMBER: 2001:886467 CAPLUS
DOCUMENT NUMBER: 136:32692
TITLE: Genetic engineering of the Trichoderma **reesei** **xylanase** II for improving thermostability and alkalophilicity for pulp bleaching and other industrial applications
INVENTOR(S): Sung, Wing L.
PATENT ASSIGNEE(S): National Research Council of Canada, Can.
SOURCE: PCT Int. Appl., 109 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2001092487	A2	20011206	WO 2001-CA769	20010531
WO 2001092487	A3	20020926		
WO 2001092487	C2	20021205		
W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG				
CA 2410917	AA	20011206	CA 2001-2410917	20010531
BR 2001011316	A	20031216	BR 2001-11316	20010531
FI 2002002120	A	20021202	FI 2002-2120	20021202
SE 2002003555	A	20030129	SE 2002-3555	20021202
SE 525279	C2	20050125		
US 2003166236	A1	20030904	US 2002-307441	20021202
PRIORITY APPLN. INFO.:			US 2000-213803P A1	20000531
			WO 2001-CA769 W	20010531

AB The present invention pertains to modified **xylanase** enzymes that exhibit increased thermostability and alkalophilicity, when compared with their native counterparts. Several modified **xylanases** exhibiting these properties are disclosed including **xylanases** with at least one modification at amino acid **position** (**10**, **27**, **29**, **75**, 104, **105**, **125**, **129**, 132, 135, **144**, 157, **161**, 162, 165) or a combination thereof. Also included within the present invention is a modified **xylanase** that comprise at least one

substituted amino acid residue and that may be characterized as having a maximum effective temperature (MET) between about 69° to about 78°, wherein the modified **xylanase** is a **Family 11 xylanase** obtained from a Trichoderma sp. The present invention also includes a modified **Family 11 xylanase** obtained from a Trichoderma sp. characterized as having a maximum effective pH (MEP) between about 5.8 to about 7.6. Modified **xylanases** characterized as having a MET between about 69° to about 78° and a MEP between about 5.8 to about 7.6 are also disclosed. The present invention is directed to the use of the modified **xylanase** as defined above in an industrial process. Also included is an industrial process, wherein the industrial process comprises bleaching of pulp, processing of precision devices, or improving digestibility of poultry and swine feed.

L10 ANSWER 8 OF 16 CAPLUS COPYRIGHT 2006 ACS on STN
ACCESSION NUMBER: 2001:567487 CAPLUS
DOCUMENT NUMBER: 135:253690
TITLE: Dissecting the Electrostatic Interactions and pH-Dependent Activity of a **Family 11** Glycosidase
AUTHOR(S): Joshi, Manish D.; Sidhu, Gary; Nielsen, Jens E.; Brayer, Gary D.; Withers, Stephen G.; McIntosh, Lawrence P.
CORPORATE SOURCE: Department of Biochemistry and Molecular Biology Department of Chemistry and The Biotechnology Laboratory, University of British Columbia, Vancouver, BC, V6T 1Z3, Can.
SOURCE: Biochemistry (2001), 40(34), 10115-10139
CODEN: BICHAW; ISSN: 0006-2960
PUBLISHER: American Chemical Society
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Previous studies of the low mol. mass **family 11 xylanase** from Bacillus circulans show that the ionization state of the nucleophile (Glu78, pKa 4.6) and the acid/base catalyst (Glu172, pKa 6.7) gives rise to its pH-dependent activity profile. Inspection of the crystal structure of BCX reveals that Glu78 and Glu172 are in very similar environments and are surrounded by several chemical equivalent and highly conserved active site residues. Hence, there are no obvious reasons why their apparent pKa values are different. To address this question, a mutagenic approach was implemented to determine what features establish the pKa values (measured directly by 13C NMR and indirectly by pH-dependent activity profiles) of these two catalytic carboxylic acids. Anal. of several BCX **variants** indicates that the ionized form of Glu78 is preferentially stabilized over that of Glu172 in part by stronger hydrogen bonds contributed by two well-ordered residues, namely, Tyr69 and Gln127. In addition, theor. pKa calcns. show that Glu78 has a lower pKa value than Glu172 due to a smaller desolvation energy and more favorable background interactions with permanent partial charges and ionizable groups within the protein. The pKa value of Glu172 is in turn elevated due to electrostatic repulsion from the neg. charged glutamate at position 78. The results also indicate that all of the conserved active site residues act concertedly in establishing the pKa values of Glu78 and Glu172, with no particular residue being singly more important than any of the others. In general, residues that contribute pos. charges and hydrogen bonds serve to lower the pKa values of Glu78 and Glu172. The degree to which a hydrogen bond lowers a pKa value is largely dependent on the length of the hydrogen bond (shorter bonds lower pKa values more) and the chemical nature of the donor (COOH > OH > CONH2). In contrast, neighboring carboxyl groups can either lower or raise the pKa values of the catalytic glutamic acids depending upon the electrostatic linkage of the ionization consts. of the residues involved in the interaction. While the pH optimum of BCX

can be shifted from -1.1 to +0.6 pH units by mutating neighboring residues within the active site, activity is usually compromised due to the loss of important ground and/or transition state interactions. These results suggest that the pH optima of an enzyme might be best engineered by making strategic amino acid **substitutions**, at positions outside of the "core" active site, that electrostatically influence catalytic residues without perturbing their immediate structural environment.

REFERENCE COUNT: 50 THERE ARE 50 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 9 OF 16 CAPLUS COPYRIGHT 2006 ACS on STN

ACCESSION NUMBER: 2000:406570 CAPLUS

DOCUMENT NUMBER: 133:189838

TITLE: Hydrogen Bonding and Catalysis: A Novel Explanation for How a Single Amino Acid **Substitution** Can Change the pH Optimum of a Glycosidase

AUTHOR(S): Joshi, Manish D.; Sidhu, Gary; Pot, Isabelle; Brayer, Gary D.; Withers, Stephen G.; McIntosh, Lawrence P.

CORPORATE SOURCE: Department of Biochemistry and Molecular Biology and the Protein Engineering Network of Centres of Excellence, University of British Columbia, Vancouver, BC, V6T 1Z3, Can.

SOURCE: Journal of Molecular Biology (2000), 299(1), 255-279
CODEN: JMOBAK; ISSN: 0022-2836

PUBLISHER: Academic Press

DOCUMENT TYPE: Journal

LANGUAGE: English

AB The pH optima of **family 11 xylanases** are well correlated with the nature of the residue adjacent to the acid/base catalyst. In **xylanases** that function optimally under acidic conditions, this residue is aspartic acid, whereas it is asparagine in those that function under more alkaline conditions. Previous studies of wild-type (WT) Bacillus circulans **xylanase** (BCX), with an asparagine residue at position 35, demonstrated that its pH-dependent activity follows the ionization states of the nucleophile Glu78 (pKa4.6) and the acid/base catalyst Glu172 (pKa6.7). As predicted from sequence comparisons, **substitution** of this asparagine residue with an aspartic acid residue (N35D BCX) shifts its pH optimum from 5.7 to 4.6, with an .apprx.20 % increase in activity. The bell-shaped pH-activity profile of this **mutant** enzyme follows apparent pKavalues of 3.5 and 5.8. Based on 13C-NMR titrns., the predominant pKavalues of its active-site carboxyl groups are 3.7 (Asp35), 5.7 (Glu78) and 8.4 (Glu172). Thus, in contrast to the WT enzyme, the pH-activity profile of N35D BCX appears to be set by Asp35 and Glu78. Mutational, kinetic, and structural studies of N35D BCX, both in its native and covalently modified 2-fluoro-xylobiosyl glycosyl-enzyme intermediate states, reveal that the **xylanase** still follows a double-displacement mechanism with Glu78 serving as the nucleophile. We therefore propose that Asp35 and Glu172 function together as the general acid/base catalyst, and that N35D BCX exhibits a "reverse protonation" mechanism in which it is catalytically active when Asp35, with the lower pKa, is protonated, while Glu78, with the higher pKa, is deprotonated. This implies that the **mutant** enzyme must have an inherent catalytic efficiency at least 100-fold higher than that of the parental WT, because only .apprx.1 % of its population is in the correct ionization state for catalysis at its pH optimum. The increased efficiency of N35D BCX, and by inference all "acidic" **family 11 xylanases**, is attributed to the formation of a short (2.7 Å) hydrogen bond between Asp35 and Glu172, observed in the crystal structure of the glycosyl-enzyme intermediate of this enzyme, that will substantially stabilize the transition state for glycosyl transfer. Such a mechanism may be much more commonly employed than is generally realized, necessitating careful anal. of the pH-dependence of enzymic catalysis. (c) 2000 Academic Press.

REFERENCE COUNT: 58 THERE ARE 58 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 10 OF 16 CAPLUS COPYRIGHT 2006 ACS on STN
ACCESSION NUMBER: 2000:300066 CAPLUS
DOCUMENT NUMBER: 133:280620
TITLE: Synthesis of thermostable **mutants** of the Trichoderma **reesei xylanase** II for pulp bleaching applications
AUTHOR(S): Sung, W. L.; Yaguchi, M.; Ishikawa, K.; Huang, F.; Wood, M.; Zahab, D. M.; White, T.; Thibault, L.; Watkinson, J.
CORPORATE SOURCE: Institute for Biological Sciences, National Research Council of Canada, Ottawa, ON, K1A OR6, Can.
SOURCE: Emerging Technologies of Pulping & Papermaking of Fast-Growing Wood, Proceedings of the International Symposium on Emerging Technologies of Pulping & Papermaking of Fast-Growing Wood, Guangzhou, Nov. 23-25, 1998 (1998), 455-461. Editor(s): Liu, Huanbin; Zhan, Huaiyu; Xie, Yimin. South China University of Technology Press: Canton, Peop. Rep. China. CODEN: 68YBAH
DOCUMENT TYPE: Conference
LANGUAGE: English
AB T. **reesei xylanase** II (TrX) has been used com. for several years in pulp bleaching applications. Here, the thermostability, temperature, and pH optima of TrX were increased by means of protein engineering. This was accomplished through the **substitution** of its (1-**29**) region with the corresponding sequence of Thermomonospora fusca **xylanase** (TfX). The resultant chimeric **xylanase** showed an improvement of +10° and +0.7 unit in the optimal temperature and pH as compared to recombinant wild-type TrX. Upstream extension from the -1 position of the new **xylanase** with the tripeptide, G-R-R, elevated the optimal. temperature and pH by 13° and 0.9 unit resp. An improvement in thermostability by 15° was also observed Three mutations (N10H, Y27M, and N29L) were identified as essential for the improvement in the chimeric **xylanase**. Several high temperature TrX **mutants** were further expressed in T. **reesei** using the promoter of the T. **reesei** cellobiohydrolase I gene. One was scaled up for commercialization. This enzyme, BioBrite HB60C, has been in use at the Weyerhauser Pulp and Paper Mill in Prince Albert, Saskatchewan, Canada since Feb. 1997. When used in the production of softwood ECF market pulp, it resulted in chemical redns. of 8.2% in ClO2 and 21% in NaOH and has saved 7.8% in bleaching chemical costs.
REFERENCE COUNT: 12 THERE ARE 12 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 11 OF 16 CAPLUS COPYRIGHT 2006 ACS on STN
ACCESSION NUMBER: 1999:259765 CAPLUS
DOCUMENT NUMBER: 131:18047
TITLE: Synthesis of thermostable **mutants** of the Trichoderma **reesei xylanase**
AUTHOR(S): Sung, W. L.; Yaguchi, M.; Ishikawa, K.; Huang, F.; Wood, M.; Zahab, D. M.
CORPORATE SOURCE: Institute for Biological Sciences, National Research Council of Canada, Ottawa, ON, K1A OR6, Can.
SOURCE: International Conference on Biotechnology in the Pulp and Paper Industry, 7th, Vancouver, B. C., June 16-19, 1998 (1998), Volume C, C61-C63. Canadian Pulp and Paper Association, Technical Section: Montreal, Que. CODEN: 67NEAW
DOCUMENT TYPE: Conference
LANGUAGE: English

AB The thermostability, temperature and pH optima of Trichoderma **reesei** **xylanase** II (TrX) have been increased by protein engineering. This was accomplished through the **substitution** of its (1-**29**) region with the corresponding sequence of the Thermomonospora fusca **xylanase** (TfX). The resultant chimeric **xylanase** showed an improvement of +10°C and +0.7 unit in the optimal temperature and pH as compared to the recombinant wild-type TrX. Upstream extension from the -1 position of the new **xylanase** with a tripeptide G-R-R, elevated the optimal temperature and pH by 13°C and 0.9 unit resp. An improvement of thermostability by 15°C was also observed Site-specific mutagenesis of the (1-**29**) region of TrX identified three mutations (Asn10His, Tyr27Met and Asn29Leu) essential for the improvement in the chimeric **xylanase**.

REFERENCE COUNT: 8 THERE ARE 8 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 12 OF 16 BIOTECHDS COPYRIGHT 2006 THE THOMSON CORP. on STN
ACCESSION NUMBER: 2005-31853 BIOTECHDS
TITLE: New nucleic acid encoding a modified **xylanase**, useful in industrial applications, for producing animal feed and human foods, aid the brewing process, and employed in decomposition of vegetative matter;
production of a recombinant glycosyl-transferase **xylanase** useful for a biocatalysis application
AUTHOR: CLARKSON K A; FENEL F
PATENT ASSIGNEE: GENENCOR INT INC
PATENT INFO: WO 2005108565 17 Nov 2005
APPLICATION INFO: WO 2004-US29575 10 Sep 2004
PRIORITY INFO: US 2003-503251 15 Sep 2003; US 2003-503251 15 Sep 2003
DOCUMENT TYPE: Patent
LANGUAGE: English
OTHER SOURCE: WPI: 2005-779481 [79]
AB DERWENT ABSTRACT:
NOVELTY - A nucleic acid encoding a modified **xylanase** comprising a polypeptide having the sequence comprising 224 amino acids (SEQ ID NO: 1), where the sequence has at least one **substituted** amino acid residue at a specific position, is new.
DETAILED DESCRIPTION - A nucleic acid encoding a modified **xylanase** comprises a polypeptide having the sequence comprising 224 amino acids (SEQ ID NO: 1), where the sequence has at least one **substituted** amino acid residue at a specific position, e.g. 2, 5, 7, 10, **11**, 16, 19, 22, 26, 28, **29**, 30, 34, 36, 38, 57, 58, 61, 63, 65, 67, 92, 93, 97, **105**, 108, 110, 111, 113, 132, 143, **144**, 147, 149, 151, 153, 157, 160, 162, 165, 169, 180, 184, 186, 188, 190, or +191. INDEPENDENT CLAIMs are also included for: (1) a modified **xylanase** comprising a polypeptide having an amino acid sequence of SEQ ID NO: 1 where the sequence has at least one **substituted** amino acid residue at a position, e.g. 2, 5, 7, 10, **11**, 16, 19, 22, 26, 28, **29**, 30, 34, 36, 38, 57, 58, 61, 63, 65, 67, 92, 93, 97, **105**, 108, 110, 111, 113, 132, 143, **144**, 147, 149, 151, 153, 157, 160, 162, 165, 169, 180, 184, 186, 188, 190, or +191; (2) a modified enzyme comprising an amino acid sequence homologous to SEQ ID NO: 1, where the sequence has at least one **substituted** amino acid residue at the specific positions defined in (1); (3) a glycosyl hydrolase of Clan C comprising an amino acid sequence homologous to SEQ ID NO: 1, where the sequence has at least one **substituted** amino acid residue at the specific positions defined in (1); (4) a modified **family 11 xylanase** comprising an amino acid sequence homologous to SEQ ID NO: 1, where the sequence has at least one **substituted** amino acid residue at the specific positions defined in (1); and (5) a family 12 cellulase comprising an amino acid sequence homologous to SEQ ID NO: 1, where the sequence has at least one **substituted** amino acid residue at the

specific positions defined in (1).

BIOTECHNOLOGY - Preferred Sequences: The **substitution** is preferably 2, 22, 28, 58, 65, 92, 93, 97, **105**, 108, **144**, 162, 180, 186, or +191. The **xylanase** has at least one **substitution** selected from H22K, S65C, N92C, F93W, N97R, V108H, H144C, H144K, F180Q, or S186C, where the **xylanase** has the following mutations: F93W, N97R and H144K; H144C and N92K; F180Q, H144C and N92C; H22K and F180Q; V108H; S65C and S186C; or H22K, F180Q, H144C andN92C. Homology to the sequence of SEQ ID NO: 1 is at least 20%. Specifically, the enzyme is glycosyl hydrolase of Clan C, a modified **family 11 xylanase**, or a family 12 cellulase.

USE - The nucleic acid and enzyme are useful in industrial applications such as the bleaching of pulp and in modification of textile fibers, for producing animal feed and human foods, and for improving properties of bread dough and the quality of bread, aiding the brewing process and are employed in decomposition of vegetative matter.

EXAMPLE - Expression vector containing cDNA-encoding **xylanase** II were used as template in the stepwise-directed mutagenesis in consecutive PCR amplifications. Synthetic oligonucleotide primers containing the altered codons for the mutations X-Y were used for insertion of the desired alteration in the native **xylanase** II primary amino acid sequence. The residues 92, 93, and **144** of the wild-type enzyme **mutants** were generated to bind the loop N143-S146 of xynII to the neighboring beta-strand. Additionally, mutagenesis was performed to generate the mutations at sites 22, 65, 97, and 108 into the **xylanase** primary sequence. Plasmid DNA from the site-directed mutagenesis PCR amplification was transformed to Escherichia coli XL-1 blue for plasmid DNA selection and transformed bacterial cells were then propagated on Luria Broth, with ampicillin 100 micrograms/ml for plasmid DNA selection and amplification of the mutated DNA. Plasmids were isolated and sequenced to confirm that they contained the desired mutations. The mutated plasmid DNA encoding the **mutant variants** was over expressed in E. coli to examine the influence of the mutagenesis on T. reseei **xylanase** Y5 **mutants** enzymatic properties.(67 pages)

L10 ANSWER 13 OF 16 BIOTECHDS COPYRIGHT 2006 THE THOMSON CORP. on STN
ACCESSION NUMBER: 2005-26923 BIOTECHDS
TITLE: New modified **Family 11 xylanase** exhibiting improved expression, for use in an industrial feed or food process, e.g. paper pulp manufacturing; vector-mediated gene transfer and expression in Trichoderma **reesei** for recombinant endo-1,4-beta-D-**xylanase** production for use in food-additive and feed addictive
AUTHOR: WHITE T; GIROUX G R; WALLACE K E A
PATENT ASSIGNEE: IOGEN BIO PROD CORP
PATENT INFO: US 2005214410 29 Sep 2005
APPLICATION INFO: US 2005-88725 25 Mar 2005
PRIORITY INFO: US 2005-88725 25 Mar 2005; US 2004-556061 25 Mar 2004
DOCUMENT TYPE: Patent
LANGUAGE: English
OTHER SOURCE: WPI: 2005-648738 [66]
AB DERWENT ABSTRACT:

NOVELTY - A new modified **Family 11 xylanase** comprising a sequence that introduces a functional consensus N-glycosylation site that is not found in the **Family 11 xylanase** from which the modified **Family 11 xylanase** is derived, where the modified **Family 11 xylanase** is ITX1, ITX2, ITX3, ITX3', ITX4, ITX4', ITX5, ITX5', Xln1-131N, or Streptomyces lividans xlnC-T131N.

DETAILED DESCRIPTION - INDEPENDENT CLAIMS are also included for: (1) a modified **Family 11 xylanase** genetic

construct comprising a promoter operatively linked to a secretion signal that is operatively linked to a coding region, the coding region comprising a functional consensus N-glycosylation site that is not found in the **Family 11 xylanase** from which the modified **Family 11 xylanase** is derived, the modified **xylanase** genetic construct resulting in an increase in expression efficiency of an encoded modified **xylanase** when compared to the expression efficiency of an encoded **Family 11 xylanase** from which the encoded modified **xylanase** was derived; (2) a genetically modified microbe comprising the modified **Family 11 xylanase** genetic construct; (3) a method of processing food or feed; and (4) a method of paper pulp manufacturing.

BIOTECHNOLOGY - Preferred Modified **Family 11 Xylanase**: The modification results from direct **substitution** of one or more amino acids within the primary sequence of the **Family 11 xylanase** from which the modified **Family 11 xylanase** is derived, where the **substitution** comprises a **substitution** of an amino acid at a position selected from position 34 (X34N), position 131 (X131N), position 180 (X180N), position 182 (X182N), or their combination, to an asparagine, the position determined from sequence alignment of the **Family 11 xylanase** with the amino acid sequence of Trichoderma **reesei xylanase** II comprising a sequence of 190 amino acids (SEQ ID NO: 1), given in the specification. The modified **Family 11 xylanase** comprises X131N. The modified **Family 11 xylanase** further comprises a **substitution** of an amino acid at a position selected from position 36 (X34NS36T), position 182 (X180N-S182T), position 184 (X182NS184T), or their combination, to a threonine. The modified **xylanase** when expressed in a Trichoderma host strain exhibits an increase in expression efficiency of at least 40% when compared to the expression efficiency of a **Family 11 xylanase** from which the modified **xylanase** is derived. The **Family 11 xylanase** is a Trichoderma **xylanase**. The Trichoderma **xylanase** is **xylanase** 1 or **xylanase** 2 from Trichoderma **reesei**. Preferred Modified **Family 11 Xylanase** Genetic Construct: The secretion signal is a Trichoderma secretion signal, where the **xylanase** secretion signal is a Trichoderma **xylanase** I secretion signal or a Trichoderma **xylanase** II secretion signal. The promoter is a Trichoderma cbh1 promoter, a cbh2 promoter, an eg1 promoter, an eg2 promoter, an eg3 promoter, an eg5 promoter, an xln1 promoter, an xln2 promoter, or a combination of two or more than two of these promoters. Preferred Genetically Modified Microbe: The microbe comprises a member of the genus of Trichoderma or Hypocrea. Preferred Method: Processing food or feed comprises treating the food or feed with an additive comprising the modified **Family 11 xylanase** above. The food or feed additive is a poultry feed additive, a swine feed additive, an additive used in baking, or an additive used in brewing. Paper pulp manufacturing comprises treating the pulp with the modified **Family 11 xylanase**.

USE - The modified **xylanase** is used in an industrial feed or food process, where the industrial process is paper pulp manufacturing (claimed).(48 pages)

L10 ANSWER 14 OF 16 BIOTECHDS COPYRIGHT 2006 THE THOMSON CORP. on STN
ACCESSION NUMBER: 2005-09577 BIOTECHDS
TITLE: Improving the alkalophilic performances of the Xyl1 **xylanase** from Streptomyces sp S38: Structural comparison and mutational analysis; plasmid-mediated **mutant xylanase** gene

transfer and expression in host cell for recombinant protein production and mutation analysis

AUTHOR: ESTEVES FD; GOUDERS T; LAMOTTE-BRASSEUR J; RIGALI S; FRERE JM
CORPORATE SOURCE: Univ Liege
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SOURCE: PROTEIN SCIENCE; (2005) 14, 2, 292-302
ISSN: 0961-8368
DOCUMENT TYPE: Journal
LANGUAGE: English

AB AUTHOR ABSTRACT - Endo-beta-1,4-**xylanases** of the **family 11** glycosyl-hydrolases are catalytically active over a wide range of pH. Xyll from Streptomyces sp. S38 belongs to this family, and its optimum pH for enzymatic activity is 6. Xynll from Bacillus agaradhaerens and Xylj from Bacillus sp. 4 1 M-I share 85% sequence identity and have been described as highly alkalophilic enzymes. In an attempt to better understand the alkalophilic adaptation of **xylanases**, the three-dimensional structures of Xynll and Xyll were compared. This comparison highlighted an increased number of salt-bridges and the presence of more charged residues in the catalytic cleft as well as an eight-residue-longer loop in the alkalophilic **xylanase** Xynll. Some of these charges were introduced in the structure of Xyl I by site-directed mutagenesis with **substitutions** Y16D, S18E, G50R,N92D, A135Q.EI39K, and Y186E. Furthermore, the eight additional loop residues of Xynll were introduced in the homologous loop of Xyll. In addition, the coding sequence of the XylJ catalytic domain was synthesized by recursive PCR, expressed in a Streptomyces host, purified, and characterized together with the Xyll **mutants**. The Y186E **substitution** inactivated Xyl I, but the activity was restored when this mutation was combined with the G50R or S18E **substitutions**. Interestingly, the E139K mutation raised the optimum pH of Xyl I from 6 to 7.5 but had no effect when combined with the N92D **substitution**. Modeling, studies identified the possible formation of an interaction between the introduced lysine and the substrate, which could be eliminated by the formation of a putative salt-bridge in the N92D/E139K **mutant**. (**11** pages)

L10 ANSWER 15 OF 16 MEDLINE on STN
ACCESSION NUMBER: 2001482607 MEDLINE
DOCUMENT NUMBER: PubMed ID: 11526340
TITLE: Oligosaccharide binding to **family 11 xylanases**: both covalent intermediate and **mutant** product complexes display (2,5)B conformations at the active centre.
AUTHOR: Sabini E; Wilson K S; Danielsen S; Schulein M; Davies G J
CORPORATE SOURCE: Department of Chemistry, Structural Biology Laboratory, University of York, Heslington, York YO10 5DD, England.
SOURCE: Acta crystallographica. Section D, Biological crystallography, (2001 Sep) 57 (Pt 9) 1344-7. Electronic Publication: 2001-08-23.
Journal code: 9305878. ISSN: 0907-4449.
PUB. COUNTRY: Denmark
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
OTHER SOURCE: PDB-1H4G; PDB-1H4H
ENTRY MONTH: 200112
ENTRY DATE: Entered STN: 20010830
Last Updated on STN: 20020122
Entered Medline: 20011213

AB The glycoside hydrolase sequence-based classification reveals two families of enzymes which hydrolyse the beta-1,4-linked backbone of xylan, **xylanases**, termed families GH-10 and GH-**11**. Family GH-

11 xylanases are intriguing in that catalysis is performed via a covalent intermediate adopting an unusual (2,5)B (boat) conformation, a conformation which also fulfils the stereochemical constraints of the oxocarbenium ion-like transition state. Here, the 1.9 A structure of a nucleophile, E94A, **mutant** of the Xyn11 from Bacillus agaradhaerens in complex with xylotriose is presented. Intriguingly, this complex also adopts the (2,5)B conformation in the -1 subsite, with the vacant space provided by the Glu-->Ala mutation allowing the sugar to adopt the alpha-configuration at C1. The structure of the covalent 2-deoxy-2-fluoroxylobiosyl-enzyme intermediate has been extended to atomic (1.1 A) resolution.

L10 ANSWER 16 OF 16 FSTA COPYRIGHT 2006 IFIS on STN

ACCESSION NUMBER: 2003:B0806 FSTA

TITLE: Specific characterization of substrate and inhibitor binding sites of a glycosyl hydrolase **family 11 xylanase** from Aspergillus niger.

AUTHOR: Tahir, T. A.; Berrin, J. G.; Flatman, R.; Roussel, A.; Roepstorff, P.; Williamson, G.; Juge, N.

CORPORATE SOURCE: Correspondence (Reprint) address, N. Juge, Inst. of Food Res., Norwich Research Park, Norwich NR4 7UA, UK. Tel. 44-1603-255068. Fax 44-1603-255038. E-mail nathalie.juge(a)bbsrc.ac.uk

SOURCE: Journal of Biological Chemistry, (2002) 277 (46) 44035-44043, 63 ref.
ISSN: 0021-9258

DOCUMENT TYPE: Journal

LANGUAGE: English

AB The importance of aromatic and charged residues at the surface of the active site of a **family 11** endo-1,4-β-**xylanase** (EC 3.2.1.8) from Aspergillus niger was evaluated using site directed mutagenesis. 10 **mutants** were expressed in Pichia pastoris, and the recombinant enzymes were characterized. Ala **substitution** of Tyr6, Tyr10, Tyr89, Tyr164 or Trp172 markedly decreased the specific activity; for Tyr6Ala and Tyr89Ala this was a result of a change in k.sub.c.sub.a.sub.t and K.sub.m, respectively, whereas for the other **mutants** a combination of increased K.sub.m and decreased k.sub.c.sub.a.sub.t was responsible. Tyr6, Tyr10, Tyr89, Tyr164 and Trp172 are proposed as substrate-binding residues, a finding consistent with structural sequence alignments of **family 11 xylanases** and with the 3-dimensional structure of the A. niger **xylanase** in complex with the modelled xylobiose. The other **variants**, Asp113Ala, Asp113Asn, Asn117Ala, Glu118Ala and Glu118Gln, retained full wild-type activity. Only Asn117Ala lost its sensitivity to **xylanase** inhibitor protein I (XIP-I) from wheat; this mutation did not affect the fold of the **xylanase**. The Asn117Ala **variant** showed kinetics, pH stability, hydrolysis products pattern, substrate specificity and structural properties identical to that of the wild-type **xylanase**. The loss of inhibition was attributed to abolition of the interaction between XIP-I and the **mutant** enzyme. A close inspection of the 3-dimensional structure of A. niger **xylanase** suggested that the binding site of XIP-I is located at the conserved `thumb' hairpin loop of **family 11 xylanases**.

L10 ANSWER 6 OF 16 CAPLUS COPYRIGHT 2006 ACS on STN
ACCESSION NUMBER: 2002:545433 CAPLUS
DOCUMENT NUMBER: 137:244453
TITLE: Evidence for temporal regulation of the two Pseudomonas cellulosa **xylanases** belonging to glycoside hydrolase **family 11**
AUTHOR(S): Emami, Kaveh; Nagy, Tibor; Fontes, Carlos M. G. A.; Ferreira, Luis M. A.; Gilbert, Harry J.
CORPORATE SOURCE: Department of Biological and Nutritional Sciences, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, UK
SOURCE: Journal of Bacteriology (2002), 184(15), 4124-4133
CODEN: JOBAAY; ISSN: 0021-9193
PUBLISHER: American Society for Microbiology
DOCUMENT TYPE: Journal
LANGUAGE: English

AB P. cellulosa is a highly efficient xylan-degrading bacterium. Genes encoding 5 **xylanases** and several accessory enzymes, which remove the various side chains that decorate the xylan backbone, have been isolated from the pseudomonad and characterized. The **xylanase** genes consist of xyn10A, xyn10B, xyn10C, xyn10D, and xyn11A, which encode Xyn10A, Xyn10B, Xyn10C, Xyn10D, and Xyn11A, resp. In this study a 6th **xylanase** gene, xyn11B, was isolated which encodes a 357-residue modular enzyme, designated Xyn11B, comprising a glycoside hydrolase **family 11** catalytic domain appended to a C-terminal X-14 module, a homolog of which binds to xylan. Localization studies showed that the 2 **xylanases** with glycoside hydrolase family (GH) **11** catalytic modules, Xyn11A and Xyn11B, are secreted into the culture medium, whereas Xyn10C is membrane-bound. xyn10C, Xyn10D, xyn11A, and xyn11B were all abundantly expressed when the bacterium was cultured on xylan or β-glucan but not on medium containing mannan, whereas glucose repressed transcription of these genes. Although all of the **xylanase** genes were induced by the same polysaccharides, temporal regulation of xyn11A and xyn11B was apparent on xylan-containing media. Transcription of xyn11A occurred earlier than transcription of xyn11B, which is consistent with the predicted mode of action of the encoded enzymes. Xyn11A, but not Xyn11B, exhibits xylan esterase activity, and the removal of acetate side chains is required for **xylanases** to hydrolyze the xylan backbone. A transposon **mutant** of P. cellulosa in which xyn11A and xyn11B were inactive displayed greatly reduced extracellular but normal cell-associated **xylanase** activity, and its growth rate on medium containing xylan was indistinguishable from wild-type P. cellulosa. Based on the data presented here, we propose a model for xylan degradation by P. cellulosa in which the GH11 enzymes convert decorated xylans into **substituted** xylooligosaccharides, which are then hydrolyzed to their constituent sugars by the combined action of cell-associated GH10 **xylanases** and side chain-cleaving enzymes.

Chk bk. refs.